

"Express Mail" mailing label No. EM057020206US

Date of Deposit: May 16, 1997

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Leslie A. Mooi

(Typed or printed name of person mailing paper or fee)

Leslie Mooi
(Signature of person mailing paper or fee)



PATENT
Attorney Docket No. 000475-205

BE IT KNOWN, that WE, DANIEL JED HARRISON, PER ANDERSSON,
PAUL LI, RODERICK SZARKA and RICHARD SMITH have invented new and useful
improvements in

MICROFLUIDIC SYSTEM AND METHODS OF USE

MICROFLUIDIC SYSTEM AND METHODS OF USE

FIELD OF THE INVENTION

This invention relates to a novel microfluidic device and methods of using this device to conduct *in vitro* studies on the reaction and effects of various compounds on cells.

REFERENCES

- The following references are cited in the application and are hereby incorporated by reference in their entirety.
1. Harrison et al., *Science*; (1993) **261**:895-897.
 2. Fluri et al., *Analy. Chem.*; (1996) **68**:4285-4290
 3. Kitagawa et al., *Electrophoresis*; (1995) **16**:1364-1368
 4. Kricka et al., *Pure and Applied Chemistry*; (1996) **68**:1831-1836
 5. Fettingner et al. *Sens Actuat B.* 17 (1993) 19
 6. Zimmerman et al., *The electromanipulation of cells*, CRC Press (1996)
 7. Fan et al., *D.J. Anal Chem.*; (1994) **66**:177-184
 8. Slappendel et al., *J.J. Blood*; (1994) **84**:904-909
 9. Agar et al., *Red Blood Cells of Domestic Mammals*; Elsevier; Amsterdam (1983)
 10. Greenwalt et al., *The human red cell in vivo*; Grune & Stratton; New York (1973)
 11. Effenhauser et al., *Science*; (1993) **261**:895-897
 12. Effenhauser et al., *Anal Chem*; (1993) **65**:2637-2642
 13. Seiler et al., *Anal Chem*; (1993) **65**:1481-1488
 14. Lehninger, A. L. *Biochemistry*; 2nd ed.; Worth Publishers: New York (1975)
 15. Berry, D. R. *The Biology of Yeast*; E. Arnold: London (1982)
 16. Rech et al., *Nucleic Acids Res*; (1990) **18**:1313
 17. Crowley, J. M. *Biophys. J.*; (1973) **13**:711-724
 18. Zimmermann et al., *Biophys. J.*; (1974) **14**:881-889
 19. Serpersu et al., *Biochim, Biophys, Acta*; (1985) 812, 779

20. Dower et al., *Nucleic Acids Res.*; (1988) **16**:6127-6145
21. Hagar et al., *Cell Calcium*; (1992) **13**:123
22. Boyum, A., "Separation of leucocytes from blood and bone marrow",
Scand. J. Clin. Invest. (1968) **21**, Suppl.97

5 **BACKGROUND**

The *in vitro* manipulation, study and processing of individual cells continues to be of importance both for theoretical evaluation as well as for the *in vitro* assaying of compounds for biological activity in such cells. However, conventional biological assay systems such as flow cytometry and cell perfusion chambers are typically operated with from 1 ml to 100 ml of reagents or more. A further disadvantage of techniques involving large volumes of cells is the inability to observe the effects on a cell both before, during and after it comes into contact with a candidate compound. Finally, statistical variations within a population of cells can limit the ability to resolve the effect of a compound.

15 Recently small disposable devices have been developed for handling biological samples and for conducting *in vitro* experiments on a controlled basis. For example, microchips have been used to electrophoretically separate mixtures of amino acids (1). Fluri et al. (2) also describe an integrated capillary electrophoresis device where electrophoresis is used to separate mixtures of amino acids.

20 The manipulation of a single cell by its electrophoretic mobility has been shown in a capillary. (3). Microchips have been designed to evaluate sperm function, principally motility, for *in vitro* fertilization. (4).

25 Analysis of the effects of candidate compounds on cell function demands careful handling of candidate compounds which are often limited in both quantity and concentration. The ability to observe the effect of the candidate compounds on individual cells in a device potentially suitable for a high level of multiplexing makes miniaturized analysis very attractive. Furthermore, the ability to observe the effect of a candidate compound on non-adherent cells would be of benefit.

Microfluidic systems embodied in a microchip would use small volumes, providing cost saving advantages for work involving expensive reagents, especially candidate compounds made for new drug screening and of course would reduce the amount of candidate compound required.

5 The ability to sort cell responses into classes and analyze each class separately would reduce the apparent statistical variation seen when large number of cells are evaluated en masse. Single cell studies would also allow the progression of events within a single cell to be evaluated, in contrast to flow cytometry where a progression of events is studied over an ensemble of cells. Statistical variations within an
10 ensemble can limit the ability to resolve a particular effect, whereas working with individual cells will maximize resolution and signal to noise for a given event.

It would be therefore advantageous to manipulate and transport cells within a microfabricated reaction device thereby allowing the observation of the cell reactions.

15

SUMMARY OF THE INVENTION

The present invention is directed to a method of observing the effect of a compound or a mixture of compounds on cells in a microfluidic device having a main flow path having a detection zone, at least two inlet flow paths intersecting and
20 merging with the main flow path at or upstream of the detection zone; applying at least one cell to a first inlet flow path and the desired compound to a second inlet flow path; inducing flow of the cells and the desired compound toward the outlet; allowing the cells to mix with the desired compound at the intersection of the second inlet flow path and the main flow path; and observing the effect of the compound on
25 the cells in the detection zone.

In one of its method aspects the present invention is directed to a method for studying calcium influx on a cell in a microfluidic system having a main flow path having a detection zone and an outlet, at least two inlet flow paths sequentially intersecting and merging with the main flow path upstream of the detection zone

applying lymphocytes to a first inlet flow path and an activator to a second inlet flow path; inducing flow of the lymphocytes and the activator toward the outlet; allowing the lymphocytes to interact with the activator at the intersection of the second inlet and the main flow path; and observing the effect of the activator on the lymphocytes

5 in the detection zone. The method wherein the device further comprises a third inlet flow path which intersects with the main flow path upstream of the detection zone and an inhibitor is added to the third inlet flow path and the effect of the inhibitor is observed in the detection zone.

In another of its method aspects the present invention is directed to a method

10 for studying leukocyte rolling comprising a microfluidic system having a main flow path with a detection zone and an outlet, at least two inlet flow paths intersecting into the main flow path upstream of the detection zone and wherein the walls of main flow path in the detection zone have attached thereto a cell adhesion molecule; applying at least one leukocyte cell to a first inlet flow path; applying a candidate compound to a

15 second inlet flow path; inducing flow of the cells and the candidate compound toward the outlet; allowing the leukocytes; candidate compound and the cell adhesion molecules to interact; and observing the leukocyte rolling in the detection zone. The method wherein the device further comprises at least two detection zones wherein the walls of the main flow path in the first detection zone are free of adhesion molecules

20 and the walls of the main flow path in the second detection zone have adhesion molecules attached thereto and observing the rolling of the leukocytes in both detection zones.

In one of its product aspects the present invention is directed to a microfluidic device comprising a main flow path comprising a detection zone and an outlet; at least

25 two inlet flow paths intersecting in fluid communication with the main flow path at or upstream of the detection zone at an upstream angle of less than 90°.

In another of its product aspects the present invention is directed to an observation device comprising a plurality of the microfluidic devices of the present

invention wherein the main flow paths of the microfluidic devices are substantially parallel at their detection zones.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a diagrammatic representation of a microfluidic device of the invention.

 Figure 2 is a diagrammatic representation of a microfluidic device with a plurality of inlet flow paths and detection zones.

10 Figures 3A, 3B and 3C are diagrammatic representations of observation devices comprising a plurality of microfluidic devices of Figure 1 illustrating that the main flow paths of the microfluidic devices are substantially parallel at their detection zones.

15 Figures 4A is a diagrammatic representation of a main flow path in a microfluidic device of the invention having weir-type trap as a flow restriction device to capture cells.

 Figure 4B is a schematic, not to scale, longitudinal cross-sectional view of the main flow path shown in Figure 4A, taken along the flow path, illustrating the effect of a weir-type trap used to capture cells.

20 Figure 4C is a diagrammatic representation of a main flow path in a microfluidic device of the invention having a weir-type trap used as a flow restriction device to capture cells. The weir-type trap defines channels which further impede the flow of cells.

 Figure 4D is an elevation of a flow restriction device as used in Figure 4C.

25 Figures 5A and 5B depict designs of the microchip COPI (Figure 5A) and PCRD2 (Figure 5B).

 Figure 6A is a photograph of yeast cells flowing through the microfluidic device.

 Figure 6B is a picture of a clump of yeast cells in the microfluidic device.

 Figure 6C is a picture of *E. coli* at an intersection of the microfluidic device.

Figures 7A and 7B are CCD images of the mixing of the Bengal Rose dye at an intersection in the microfluidic device. Figure 7A depicts the flow of the fluid; Figure 7B depicts the mixing of fluid when the flow is stopped.

Figure 8 is a schematic of the design of the PCRD1.

5 Figure 9 is a schematic of the design of the PCRD3.

Figures 10A, 10B and 10C illustrate the lysis of canine erythrocytes when SDS is mixed with erythrocytes at an intersection of the microfluidic device.

Figure 11 is a graph of a kinetic plot of the calcium flux in a human lymphocyte.

10

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a microfabricated device for the evaluation of the effect of one or more compounds or a mixture of compounds on individual cells. The invention further provides systems which include the microfabricated device of the invention together with a detection device, e.g. an microscopic device.

15

Definitions

However, prior to discussing this invention in further detail, the following terms will first be defined.

20

The term "flow paths" means the capillary paths or channels that are present in the microfluidic device.

25

The term "flow inducing means" encompasses devices which have the ability to induce flow. Flow may be induced by negative pressure, positive pressure, electrophoretically or osmotically. This may be accomplished by peristaltic or reciprocating pumps, syringes, electricity, or piezo elements. In a preferred embodiment, the flow inducing means includes the ability to reverse the flow direction. For example, a device capable of exerting both positive and negative pressure can induce flow by positive pressure and can reverse flow by negative pressure.

The term "stop flow" means stopping or temporarily halting the fluid flow through the microfluidic device so as to allow the mixing of the contents of two or more flow paths and so that observation of the reaction of the candidate compound on the cell is possible.

5 The term "candidate compound" or "proto-drug" or "desired compound" means drug candidates, proto-drugs, cell inhibitors, cell activators, cell adhesion molecules including selectins, lectins, integrins, and cell receptors, and any other compound or reagent of interest in determining its affect upon a cell.

10 The term "cell" includes both eukaryotic and prokaryotic cells, including bacteria, yeast, mammalian cells etc. Preferably the cells are eukaryotic cells and more preferably they are leukocytes. Other cells include gangliocytes, fibroblasts or any cell which can be suspended in a single cell suspension.

15 The term "cell adhesion molecule" means any molecule which facilitates cell adhesion. These include selectins, lectins, integrins and any other cell surface molecule which will facilitate adhesion of leukocytes to the walls of the device.

 The term "activator" means a candidate compound believed to induce a cell to respond. In a preferred embodiment the activator is the calcium ionophore, A23187 (calcimycin).

20 The term "inhibitor" means a candidate compound believed to inhibit a cell from responding to an activator compound.

 The term "calcium influx" describes the process of induction of intracellular Ca^{2+} flux within leukocytes, which is an indicator of cell activation. Activated leukocytes are directly involved in normal and abnormal immune response. A rapid increase in the intracellular messenger, Ca^{2+} , is the second signal in the activation pathway of all mammalian cells. Thus, a rise in cytosolic free Ca^{2+} is the key
25 signaling event for activation.

 The term "leukocyte rolling" means the process of a leukocyte rolling on an endothelium as the first event leading to cell migration through tissue. The basic molecular mechanisms of the inflammatory response comprise a cascade of events

brought about by the sequential binding of different adhesion receptors. The first step in the adhesion cascade is the reversible binding mediated by selectins which cause the leukocytes to roll along the inflamed endothelium. The second step is leukocyte activation mediated by cytokines that induce leukocytes to flatten on the endothelium resulting in transmigration into the tissue. Cells which are activated and adhere to a surface appear to travel in a rolling fashion in a flowing stream, exhibiting a lower average velocity than nonadhering cells.

With reference to Figure 1, the microfluidic device of the present invention comprises a main flow path 2 having an outlet 4 and a detection zone 10 positioned so as to allow detection of any cellular reaction and at least two inlet flow paths 8 and 8', in fluid intersection with the main flow path 2 upstream of the detection zone 10 and the outlet. The inlet flow paths have inlet ports 6.

The microfluidic device is obtained from a solid substrate, preferably in the form of a chip. The dimensions of the device, e.g., chip, are not critical but preferably these dimensions are in the order of about 0.001 centimeters to about 10 centimeters thick and approximately about 0.3 centimeters to about 30 centimeters on a side.

The device of the invention may be conveniently constructed by forming the flow passages in the surface of a suitable substrate or base plate and then mounting a cover over such surface. It is also contemplated that the invention may be constructed by forming the flow passages in the surface of the base and the cover and then aligning the passages when mounting the cover onto the base.

The base and the cover may comprise a material such as silicon, polysilicon, silica glass, thermocouple materials, gallium arsenide, polyamide, silicon nitride and silicon dioxide. The cover and/or base may also comprise a plastic material, such as acrylic, polycarbonate, polystyrene, polyethylene, polydimethyl siloxane or other resin materials. Preferentially the cover and base may comprises a material that permits detection of a signal, more preferably it is a material transparent to ultraviolet, infra red or visible light or permits radioactive detection. In a particularly preferred

embodiment it may be a relatively thin, fusion or anodically bonded layer of glass or ultrasonically welded plastic sheet material.

5 The flow paths 2, 8 and 8' have dimensions of from about 0.1 μm deep by 0.1 μm wide to about 1 mm deep by 2 mm wide. Preferably, the flow paths are from about 5 μm deep by 15 μm wide to about 30 μm deep by 90 μm wide. The width of the flow paths in the device is sufficiently small to enable observation of the effect of a candidate compound on a single cell. Preferably, the length of the flow paths will be within the range of from about 50 μm to about 2 meters, preferably they are from about 1 millimeter to about 100 centimeters in length.

10 The flow passages and other structures, when viewed in cross-section, may be triangular, ellipsoidal, square, rectangular, circular or any other shape at least one cell-sectional dimension of which, transverse to the path of flow of sample fluid through or into a given structure, is from at about 0.1 μm to about 2 mm, preferable from about 5 μm to about 90 μm .

15 In a preferred embodiment one or more inlet flow paths are of greater diameter than the main flow path. This difference in diameter between the inlet flow paths and the main flow assists in the regulation of the flow rates. For example, when the flow inducing means is electricity, the difference in flow path widths may compensate for the drop in electrical potential in the main path and may achieve a better potential gradient along the main flow path.

20 The inlet paths intersect with the main flow path. With reference to Figure 1, the upstream angle of intersection of each of the inlet flow paths with the main flow path is preferably at an angle less than 90° , more preferably at an angle of less than about 70° and most preferably at an angle of less than 50° . It has been found that where the inlet flow path with the main flow path intersects at an upstream angle of 90° or greater the turbulence generated at the intersection of the inlet flow path with the main flow path may result in damage or rupture of the cells. Such damage or rupture is to be avoided if an accurate determination of the effect of the candidate

compound is to be determined. In addition, lysis of the cells may cause the main flow path to become clogged, preventing subsequent observations of additional cells.

It is contemplated that excess sample fluid, cells, reagents, wash solutions and the like from the main flow path outlet 4 may be routed into a waste receptacle of
5 adequate capacity such that all sample fluid and reaction products are safely contained for disposal.

The effect of the candidate compound on the cell is monitored through the detection zone 10. Detection of the effect of the candidate compound on the cell can be detected by any number of methods including by optical detection through a
10 transparent cover or a translucent section of the base either visually or by machine. The effect of the candidate compound can also be monitored by changes in flow properties or in electrical conductivity. Devices such as valves, pressure sensors, and other mechanical sensors can be fabricated by well established technologies. Such devices can be manufactured directly on a silicon substrate according to well
15 established technologies.

The device also may be utilized in combination with an appliance for viewing the effect of the candidate compound on the cells. The appliance in one embodiment may comprise a microscope for viewing the effect. It is contemplated that the microscope may be an inverted microscope or a confocal microscope. A UV light
20 source or laser beam used to activate cell fluorescence may also be used to observe the effects of candidate compounds on a cell.

In another embodiment, a camera may be included in the appliance. The camera could include a video camera. In another embodiment the data may be observed using a photomultiplier tube (PMT) affixed to the microscope instead of a
25 camera. If a PMT is utilized, it is contemplated that a pinhole will be used in conjunction with the microscope to limit the field of view of the PMT.

It is contemplated that a scintillation device may also be used to detect the effects of a candidate compound on a cell where a radioactive dye is utilized.

In another embodiment, electrical conductors may be fabricated in the base of the device to enable transmission of signals. The electrical conductors in the device carry signals from pressure or electrical conductivity sensors enabling detection of the conductivity or pressure of the cells in the flow system.

5 It is contemplated that the inlet flow paths have inlet reservoirs for placing the cells and or the candidate compounds into the inlet flow paths.

The microfluidic device can be used in combination with an appliance for delivering fluids and cells to the microfluidic device via the inlet ports 6 and 6' and discharging fluid and cells from the device via the outlet 4. The appliance may
10 include an implement such as a pump for conveying the fluids and cells through the flow paths of the microfluidic device. The pump may be incorporated into the device according to known microfabrication techniques.

Fluid control can be achieved using various pumps, such as micromachined pumps, reciprocating pumps, peristaltic pumps, diaphragm pumps, syringe pumps,
15 volume occlusion pumps as well as endosmotic induced flow, flow induced by electrochemical evolution of gases and other pumping means known to those skilled in the art. Fettinger et al.(5) have shown it is possible to use external pumps to control a valveless, microfluidic system. It is contemplated that some reservoir contacts to the inlet flow paths 6 and 6' would be left open to atmosphere, while others were
20 connected to pumps operated in a combination of pressure and suction modes to allow the control of the flow at the intersections of the paths. Operating with pumps in a negative pressure mode will facilitate small proto-drug reservoir volumes, in the range of from about 10 μ l to about 200 μ l. It is contemplated that the accuracy and sensitivity of this fluid control method can be enhanced by designing the size of the
25 flow channels to restrict flow where desired and minimize flow resistance where necessary. Good fluid control can be achieved without internal valves in the microchannels. Syringe pumps are preferred since the flow rate and bulk volume are desirable. Alternative pumps are microfabricated pumps, such as the HSG-IMT VAMP (Villengen, Germany) or other commercially available micropumps.

Flow of the fluid within the microfluidic device may also be controlled using electrical fields. In uncoated glass microchips, the solvent mobility due to electroosmotic flow is greater than the electrophoretic mobility of the cells, so the net flow direction of the cells is toward the cathode at near-physiological pH values.

5 High electric fields, in the range of 1 kV/cm for yeast cells, 2-4 kV/cm for human erythrocytes and 5-10 kV/cm for yeast cells have previously been used to introduce DNA or other labeled substances into these cell types via electroporation. Fields of those magnitudes caused membrane permeation but did not result in cell lysis. Preferably the fields are less than about 600V/cm and more preferably less than 100
10 V/cm.

It is contemplated that such means for inducing the flow of the candidate compound and the cells through the inlet flow paths and into the main flow path may also be used to stop the flow of such compounds and cells such that one or more cells are present in the detection zone simultaneously with or shortly after the candidate
15 compound has interacted with the cell. Stoppage of the flow will allow observation of the complete effect of the candidate compound on the cell.

Means for regulating the temperature in the main flow channel 10 may optionally be utilized to enhance the reaction conditions. Means for sensing the temperature in the main flow passage may also be provided, if desired. The
20 temperature sensing means may be operatively connected to a microprocessor or similar device which controls the overall function of the system so as to correlate the sensed temperature change with the interaction of the cell with the candidate compound.

With reference to Figure 2, it is contemplated that the microfluidic device of
25 the present invention may comprise a plurality of inlet flow paths 8, 8', 8'' and 8''' in fluid communication with the main flow path. It is also contemplated that the microfluidic device may contain a plurality of detection zones 10, 10' and 10'' each one placed adjacent to or immediately downstream of the intersection of the inlet flow path with the main flow path. Such a device would allow the observation of a

number of different candidate compounds on a single cell as the cell flowed downstream in the main flow path. Alternatively, such a device would allow the simultaneous monitoring of different candidate compounds on different cells as the cells intersect with the compounds along the main flow path.

5 It is also contemplated that the detection zone may be located both upstream and downstream of the intersection of an inlet flow path with the main flow path. This would allow the observation of a single cell both before and after exposure to the candidate compound or alternatively the simultaneous observation of multiple cells before and after exposure to the candidate compound.

10 Figures 3A, 3B and 3C illustrate other embodiments of the present invention in which multiple microfluidic devices make up observation devices **19**, **39**, and **51** suitable for practicing the methods of the invention. Such devices may be used to study leukocyte rolling. In such observation devices, multiple channels are used to observe at least three relevant parameters of studying leukocyte rolling: velocity in
15 absence of rolling, velocity on a surface that induces rolling, and velocity on the same surface with drug present. A microscope can simultaneously observe all of the channels at once.

 Figure 3A depicts a contemplated arrangement for an observation device **19** with multiple main flow paths on a single chip. This allows simultaneous testing of
20 several candidate compounds on the same type of cell at one time. Cell chamber **20** is an inlet which is in fluid connection with microchip channels **22-22'''** which define four main flow paths. Test chambers **24-24'''** feed through additional inlets which intersect microchip channels **22-22'''**, respectively, downstream from cell chamber **20** and add a suitable candidate compound for reaction with the cells flowing from cell
25 chamber **20**. Flows from activator chambers **26** and **26'''** intersect microchip channels **22** and **22'''**, respectively, downstream from the point of addition of the suitable candidate compounds from test chambers **24** and **24'''**. Each of the microchip channels **22-22'''** pass through an observation zone **28** and flow into a common waste chamber **30**. The flow paths may be arranged so that the observation

zone (microscope view) **28** allows simultaneous observation of the four main flow paths of the microchip channels **22-22'''**.

As illustrated in device **39** in Figure 3B, multiple microfluidic devices on one chip allow multiple tests at one time. These multiple tests can include a control, on the same chip but in separate devices. In device **39** microchip channels **42-42'''** define main flow paths from separate cell chambers **40-40'''**, respectively. Flows of candidate compounds from test chambers **44-44'''** intersect microchip channels **42-42'''**, respectively. Activator chambers **46-46'''** intersect microchip channels **42-42'''** downstream from the point of addition of the candidate compounds. Each of the microchip channels **42-42'''** pass through an observation zone **48** and flow into a common waste chamber **50**. As is apparent in Figure 3B, a microscope view **48** observes the microchip channels **42-42'''** at a point downstream from the point of addition of activator.

Figure 3C shows yet another observation device **51** providing an arrangement for multiple separate microfluidic channels on a single microchip. Microchannels **50-50''** define three main flow paths from cell chambers **52-52''**, respectively. Downstream are inlet flow paths **53-53''** from candidate compound reservoirs **54-54'**, respectively. Still further downstream, the three main flow paths **50-50''** pass through a detector **60**, such as a microscope, in which the cell rolling characteristics can be observed. Finally, the microchannels flow into outlets **58-58''**.

Device **51** is provided with additional fluid paths **57-57''** which connect to fluid flow controllers **56-56''**, respectively. These fluid flow devices can induce positive or negative pressure upon the flows in main flow paths **50-50''**. Once the desired cells, for example leukocytes, are loaded into the cell chambers **52-52''** and candidate compounds are loaded into the reservoirs **54-54'**, flow may be induced by negative pressure applied by flow controllers **56-56''**. The flow may be stopped and/or reversed by applying a positive pressure via the flow controllers **56-56''**. Observation of the channels may be done as earlier described.

It is contemplated that different combinations of test compounds and activators or inhibitors may be simultaneously tested in each of the Figures 3A, 3B and 3C.

Another embodiment of the present invention, which is illustrated in Figures 4A and 4B, utilizes changes in flow path cross-sectional area to provide a cell capture zone. This variation can take the form of a weir-type trap. The main flow path **2** at the point of intersection with an inlet flow path **8** contains two barriers (weirs) **12** proceeding across the bottom **14** of the main flow path on either side of the inlet flow path. The barriers are not of sufficient height to reach the top **15** of the main flow channel. The barriers or weirs form a cell reservoir **13** such that cells introduced into the inlet flow path **8** tend to be captured in the cell reservoir **13**. Any buffers or candidate compounds introduced into the main flow path upstream of the weirs or barriers will flow over the barriers and interact with the captured cells **16**. It is contemplated that instead of using weirs or barriers to alter the flow path, the section of the main flow path immediately adjacent to the inlet flow path **8** may be deeper or the bottom **14** of the main flow path may be lower than the bottom of the inlet flow path or the main flow path on either side, effectively creating the cell reservoir **13**. It is further contemplated that a cell capture force may be applied at the tops **15** of the barriers or weirs **12** or at the edge of the cell reservoir. Such cell capture force could take the form of electrical currents induced by electrodes as is done in dielectrophoresis. (6)

A variation of the cell-reservoir forming barriers (weirs) **12** is illustrated in Figures 4C and 4D. With reference to Figure 4C, the weirs contain channels **18** proceeding in the direction of the flow path. Such channels **18** may be triangular in cross-section. There is a second inlet flow path **8'** downstream of the downstream weir **12'**. It is contemplated that a chemotactic agent may be introduced into the flow path via inlet flow path **8'** immediately downstream of the weir **12'**. Lymphocyte reaction to the chemotactic agent causes the lymphocyte to migrate through the channels **18** of the weir **12'** toward the chemotactic agent.

Methodology

The microfluidic device described above can be used in a method for observing the effect of a compound on a cell, as will be described in further detail below.

5 With reference to Figure 1, a solution containing the specific cells to be observed is introduced into the first inlet flow path **8** via the inlet port **6**. The candidate compound is introduced into the second inlet flow path **8'** via a second inlet port **6**. Both of the inlet flow paths **8** and **8'** are in fluid communication with the main flow path **2**. Flow of the cells and the candidate compound toward the main
10 flow path is induced. The cells are allowed to interact with the candidate compound at the intersection of the second inlet flow path **8'** and the main flow path **2** and the effect of the compound on the cells is observed in the detection zone **10**. It being understood that the order of the intersection of the first and second inlet paths with the main path flow **2** is not determinative. The cells may be introduced into the second
15 inlet flow path **8'** and the candidate compound introduced into the first inlet flow path **8**.

After the cells and/or compounds are loaded, flow is induced within the microfluidic device. In a preferred method, the flow is controlled by a syringe or a syringe pump attached to the outlet **4** of the main flow path **2**. The syringe is drawn
20 so as to place a negative pressure on the system and induce flow. Flow may also be induced with electricity or by a piezoelectric element by methods known in the art.

The microfluidic device has at least one detection zone where the contents of the capillary flow paths may be observed.

It is contemplated that the flow of the cells and the candidate compound may
25 be stopped when a cell or group of cells enters the intersection of the main flow path and the second inlet path so as to observe the effect of the candidate compound on the cells. The length of time that the flow is stopped or interrupted will be dependent upon the length of time for the reaction of the cell with the compound. The flow is halted to allow the cell to react sufficiently to the compound such that a possible

change in the cell or the cellular function may be observed. Such a period of time is preferably from 0.1 seconds to 60 minutes, more preferably from 1 second to 10 minutes. Once the cell has reacted to the compound, if such a reaction occurs, or the cell and compound have mixed for sufficient to time to allow a reaction to occur, flow
5 of the cell and the compound is again induced to flush the cell from the detection zone and allow the observation of another cell with the candidate compound.

It is contemplated that the microfluidic device may contain a plurality of inlet flow paths as depicted in Figure 2. In such a device cells and buffer are loaded into one inlet path, the candidate compound is loaded into a second inlet path and a second
10 compound may be added into a third inlet path. Flow of the cells and the compounds into the main flow path and toward the outlet is induced and the effect of the compounds on the cells is observed in the detection zone. It is contemplated that the first compound may be an inhibitor and the second compound may be an activator of a cell function.

15 In a preferred method, the cells and inhibitor are allowed to mix within the main flow path. The flow is controlled with the syringe or syringe pump so that the cells and inhibitor mix as they travel through the main flow path. The cell/inhibitor mixture then contacts the activator at the intersection of the third inlet. Back pressure on certain inlet flow paths may also be used in this method. A syringe is placed on
20 the inlet path while the syringe on the outlet is drawn. The open inlet flow paths (those without a syringe attached) will flow more easily toward the outlet. This allows for a controlled mixing of inlet paths into the main flow path.

Reagents may be added to the main flow path to facilitate observation of the cell reaction to the candidate compound. Such reagents include visible dyes,
25 fluorescent dyes and radioactive dyes. Such dyes may react directly with components produced by the cells in reaction to the candidate compound. Alternatively, such dye may be bound either covalently or non-covalently to a ligand or antibody which binds to components produced by the cells in reaction to the candidate compound. Radioactive dyes include ^{32}P , ^{125}I and ^3H . Fluorescent dyes include fluorescein,

calcein-AM, Fluo-3, Fura-2, Indo-1, Quin-2 and related compounds available from Molecular Probes. Fluorescent pH indicators include compounds such as SNAFL, SNARF and related pH indicators. Cell viability may be measured using the compound calcein-AM. DNA may be detected in dead cells with ethidium
5 homodimer.

The walls of the flow paths may also be coated prior to the introduction of cells and/or compounds into the device. Compounds which are useful include fetal calf serum, bovine serum albumin, protein not related to the compounds being studied, gelatin or ovalbumin. In a preferred embodiment the coating on the walls of
10 the flow paths is electrostatically neutral to reduce the electrostatic charge of the walls of the flow paths. Preferably, fetal calf serum is used to coat the flow paths. A polymer coating such as polysiloxane or polyacrylamide or polymethylmethacrylate may also be used to coat the flow paths prior to using the device.

The device of the present invention may be utilized to examine the induction of
15 intracellular Ca^{2+} flux within leukocytes, which is an indication of cell activation. Activated leukocytes are directly involved in normal and abnormal immune responses. A rapid increase in the intracellular messenger Ca^{2+} is the second signal in the activation pathway of all cells. Similarly with neutrophils a tenfold increase in the concentration of cytosolic free Ca^{2+} is the key signaling event of activation. The
20 methods of this invention contemplate evaluating the influence of agonists or antagonists on cell function by this method.

Drugs which prevent Ca^{2+} activation can be of considerable importance in treating abnormal immune responses. Screening of drug candidates, or protodrugs, by evaluating their effect on Ca^{2+} channel activation of non-adherent lymphocytes is
25 useful. The ability to determine the kinetics of proto-drug binding and release by cells, while not replacing conventional pharmacokinetic studies, would provide additional, highly informative data in the early stages of screening.

In an assay to measure Ca^{2+} influx, the cells and buffer are mixed with a calcium indicator such a Fluo-3 and applied to the first inlet path, the activator is

applied to the second inlet path and flow of the cells and the activator is induced. The flow is stopped when one or more cells mix with the activator at the intersection of the main flow path and the second inlet flow path. Any Ca^{2+} influx is measured in the detection zone.

5 The device of the present invention may also be used to evaluate proto-inhibitors on the calcium influx. It is contemplated that the proto-inhibitor may be mixed with the cells, buffer and calcium indicator prior to applying the cells to the first inlet flow path. The activator is applied to the second inlet path and flow of the cells and the activator is induced. The flow is stopped when one or more cells mix
10 with the activator at the intersection of the main flow path and the second inlet flow path. Any Ca^{2+} influx is measured in the detection zone. It is also contemplated that a device containing three inlet flow paths may be used. The cells and buffer are mixed with a calcium indicator such as Fluo-3 and applied to the first inlet path, the proto-inhibitor is added to the second inlet flow path, flow is induced and the inhibitor
15 and the cells are allowed to mix. The flow may be stopped to allow sufficient time for the cells to react with the inhibitor. The activator is applied to the third inlet path and flow of the cells and the activator is induced. The flow is stopped when one or more cells mix with the activator at the intersection of the main flow path and the second inlet flow path. Any Ca^{2+} influx is measured in the detection zone.

20 The method may also be used to study oxidative bursts of cells. The enzyme NADPH oxidase, present in stimulated granulocytes is involved in the multicomponent enzyme pathway that results in the production of superoxide anion O_2^- . The oxidative burst is an important bactericidal property of these leukocytes. However, under pathologic conditions, this oxidative burst may contribute to tissue
25 injury. The enzyme pathway can be activated by a number of antagonists including whole bacteria, phorbol esters, fMLP (formyl methionine-leucine-phenylalanine) chemotactic peptide and a variety of cytokines. The production of O_2^- can be measured using the oxidant-sensitive fluorescent dye dihydrorhodamine 123 in the manner similar to that described above for measurement of Ca^{2+} influx.

The methods of this invention may also be used to study leukocyte rolling on an adhesion matrix. The rolling of leukocytes on an endothelium is recognized as the first event leading to cell migration through tissue, a key event in autoimmune diseases such as arthritis. Inhibition of the primary event of cell rolling, i.e. selectin-
 5 ligand interaction, should block a number of biologically important events.

In the methods of the present invention, a purified cell adhesion molecule is introduced into the main flow path and flow of the cell adhesion molecule down the main flow path, through the detection zone, is induced to allow the cell adhesion molecules to adhere to the walls of the main flow path in the detection zone. The
 10 leukocytes are introduced into the first inlet flow path and induced to flow into the main flow path. Any rolling of the leukocytes upon exposure to the adsorbed cell adhesion molecules is observed in the detection zone. It is contemplated that an inhibitor or activator of rolling could be further introduced into the device of the present invention through a third or fourth inlet flow path and the effect of this
 15 inhibitor or activator on the rolling of the leukocytes observed in the detection zone. Preferably, this method is conducted using the device illustrated in Figure 3 to allow observation of different cell adhesion molecules, inhibitors and activators on various cells simultaneously.

The present invention provides several advantages over the currently available
 20 methods to study the effects of compounds on cells. Currently, bioassays for calcium flux are performed on microtiter plates or with cuvette mixing. Bioassays for leukocyte rolling are performed in animals or in large rolling chambers. This invention allows real time viewing of the effect of a compound of interest on a living cell. The size of the microfluidic device leads to an increased number of compounds
 25 that can be physically studied. The methods of the present invention decrease the amount of sample compound needed and allows easy manipulation of individual or small clumps of cells.

In order to further illustrate the present invention and advantages thereof, the following specific examples are given, it being understood that the same are intended only as illustrative and in nowise limitative.

5

EXAMPLES

Example I - Preparation of the Microchip Device

10 Glass devices (3 in. x 3 in.) were fabricated at the Alberta Microelectronic
Centre, using a modified silicon micromachining technique. (7) The substrate was a
600- μm -thick 0211 glass plate (Corning Glass Works, Corning, NY). Channels of
either 15 or 30 μm depth were etched on one glass plate. The lengths and widths of
the channels are depicted in Figures 5A and 5B. A cover plate, with holes drilled for
15 external access was thermally bonded to the etched plate using the following
temperature program: 0.5 h at 440°C, 0.5 h at 473°C, 6 h at 605°C, 0.5 h at
473°C, followed by cooling overnight. Small plastic pipet tubes, glued around the
drilled holes using epoxy resin, were used as device reservoirs to contain sample or
buffer solutions. Pt wires were inserted for electrical contact. Some chips were
20 coated internally using AquaSil (Pierce, Rockford, IL). A 1% aqueous solution was
forced into the channels by syringe, removed under vacuum, and then allowed to cure
overnight.

Prior to bonding the plates together, both plates were pressure washed in a
class 100 clean hood with MicroAutomation 2066 high-pressure cleaning station. The
25 two plates were aligned before contacting. Once contacted, enough pressure was
applied to drive out all the trapped air. Good contact was evidenced by the absence
of interference fringes. Small particles of dirt resulted in appearance of Newton's
rings around the contaminant, necessitating separation and recleaning of the wafers.

This cleaning method was also applied to Pyrex wafers, resulting in so few bonding defects that only one bonding cycle was required.

Example II - Electrophoretic Mobility in a Microchip

5 Buffer Solutions and Reagents

A phosphate buffer, 40 mM each of Na_2HPO_4 and KH_2PO_4 (BDH analytical grade) was adjusted to pH 7.4 with either NaOH or HCl and used as an isotonic buffer for canine erythrocytes. (The plasma concentrations of Na^+ and K^+ in canine plasma are 94 and 6 mM. respectively. (8,9) Assuming a monoanion as the
10 counterion gives as osmolarity of 200 mM, equal to that of the buffer prepared.) A hypotonic solvent, deionized water, was used to prepare yeast or *E. coli* cell suspensions. Sodium dodecyl sulfate (SDS, Serva, analytical grade) was prepared at 3 mM (0.1 wt %) in deionized water. Other chemicals were reagent grade and were used without further purification.

15

Cell Samples

Red blood cells of a healthy dog were obtained from Heritage Medical Research Centre (Edmonton, AB, Canada). Blood samples were collected using EDTA as the anticoagulant. These were centrifuged for separation into various blood
20 components. After removal of the plasma and buffy coat, the erythrocytes were isolated as a cell pellet. A citrate-phosphate-dextrose (CPD) solution, (9), used to store human whole blood for enhancement of the poststorage viability of red cells, (10), was adapted for handling and storing the canine cells by addition of KCl and NaCl as well. The solutions contained 2.5 mM dextrose, 1.6 mM sodium citrate,
25 0.28 mM citric acid, 0.15 mM Na_2HPO_4 , 94 mM NaCl, and 6.0 mM KCl. An erythrocyte suspension of 5% hematocrit was used. The cells were washed several times with the isotonic phosphate buffer to remove the storage buffer, using a Sanyo MSE MicroCentaur centrifuge.

Baker's yeast type II (*Saccharomyces cerevisiae*) was obtained from Sigma (Milwaukee, WI). *E. coli* (nonpathogenic strain, BlueScript) was donated by D. Khasa of Renewable Resources, University of Alberta. Yeast cell counts were 10^8 /ml, and *E. coli* cell counts were 3×10^8 /mL, unless stated otherwise.

5 Figures 5A and 5B illustrate the layout of the device designs used for this work. Both devices utilize a "double-T" structure, which allows for formation of geometrically defined plugs of solution. (1, 11, 12). Potentials were applied to the various reservoirs to direct solvent flow within the four intersecting channels.

10 The experiment for yeast cell transport was performed with the COPI device, illustrated in Figure 5A. During loading of yeast cells, the sample reservoir (S) was at +100 V, while the sample waste (SW) reservoir was at ground. During injection, the buffer reservoir (B) was set at -500 V, the S and SW reservoirs were both at +450 V, and the buffer waste (BW) reservoir was at ground.

15 The experiment for *E. coli* cell transport was performed at the Y-intersection of the PCRD2 device, illustrated in Figure 5B. The cells were introduced at the BW1 reservoir whose potential is at ground. The BW2 reservoir was at -200 V. The potentials of the other reservoirs (B, S, and SW) were floating initially. To control flow direction, -200 V was then toggled back and forth between BW2 and SW reservoirs, leaving the alternate reservoir floating.

20 The visual cell lysis experiment was performed in the COPI device (Figure 5A). The B reservoir, which contained erythrocytes and the S reservoir, containing 3 mM SDS, were both at +150 V, while the SW reservoir was at ground. The potential at BW was floating.

25 For the cell lysis experiment using PMT detection, the PCRD2 device (Figure 5B) was used. Cells were introduced into the S reservoir to which 400 V was applied, versus ground on the SW reservoir. A 3 mM SDS solution was introduced into the B reservoir. During cell lysis, 400 V was applied to both reservoirs S and B, while lysis was terminated by leaving reservoir B floating. The other reservoirs, BW1 and 2, were left floating at all times.

Observations were performed with an Olympus microscope (BH-2) equipped with a JVC video camera (TK-1280). The images were first recorded using a JVC S-video cassette recorder (HR-S7200U) and then captured using a Computer Eyes/1024 frame grabber board and printed with a Codonics NP1600 photographic printer.

5 Alternatively, an adapter was made to position a photomultiplier tube (PMT) on the microscope to detect the passage of cells via the induced light scattering. A pinhole 10 μm in diameter was used to limit the PMT's field of view. The computer controlled system for application of electric voltages at the device reservoirs and for recording the PMT signal has previously been described. (7, 13).

10 The three cell types studied are quite varied in size and shape: baker's yeast is close to spherical, with about a 5- μm diameter; the *E. coli* strain used is tubular, with the long dimension varying from submicrometer to about 2 μm while canine erythrocytes (red blood cells) are 8 μm in diameter and 2 μm thick. (9, 14, 15) These cell types are all negatively charged, (9, 14, 15), so in an electric field they
 15 will migrate in the direction of the anode due to electrophoretic effects. However, in uncoated glass chips, the solvent mobility due to electroosmotic flow is greater than the electrophoretic mobility of the cells, so the net flow direction of the cells is toward the cathode at near-physiological pH values. High electric fields in the range of 1 kV/cm for yeast cells, (16). 2-4 kV/cm for human erythrocytes, (17, 18, 19),
 20 and 5-10 kV/cm for yeast cells, (20), have been used to introduce DNA or other labeled substances into these cell types via electroporation. Fields of those magnitudes caused membrane permeation but did not result in cell lysis. Fields of less than 600 V/cm and more typically 100 V/cm were used, so that no lysis should occur. However, the small number of cells that were directly in the double-layer
 25 region of the electrodes used to deliver the driving potential in the reservoirs did lyse.

Since electroosmotic flow follows the electric field lines, application of a potential between sample and sample waste reservoirs caused the cells to negotiate the corners of the intersections in the double-T layout and form a plug of cells along the axis of the main channel.

Figure 6A confirms that this valveless control scheme for directing cell transport is successful. A potential of 100 V (60 V/cm in the double-T region) produces a stream of yeast cells moving through the double-T with an average velocity of 0.18 ± 0.02 mm/s. Once a plug of yeast cells is formed under steady state conditions at the intersection, it can be injected down the main channel by switching the potentials to the buffer and buffer waste reservoirs. Figure 6B illustrates a packet of six cells formed in this fashion, beginning to migrate along the main channel. Potentials of 500 V at B and 450 V at both S and SW gave an electric field in the main channel of 60 V/cm and a velocity of 0.16 ± 0.02 mm/s. With the potential at the injector intersection at about 455 V, these polarities ensured weak back flow into the sample and sample waste reservoirs during injection, to help define the cell packet. Fields at least as high as 160 V/cm can be applied to give velocities of 0.49 ± 0.08 mm/s for the yeast cells.

Figure 6C illustrates an example of the manipulation of *E. coli* cells at the Y-intersection of the PCRD2 device, using electric fields to control the direction of flow. These cells moved with an average velocity of 0.054 ± 0.005 mm/s at 35 V/cm, and velocities of 0.28 ± 0.05 mm/s were obtained at 140 V/cm. Higher voltages give higher flow rates without cell lysis but were not studied in detail, since the video rate of the camera did not allow us to resolve the cells at higher velocities. The flow direction could be readily switched between the two branches of the Y by switching the negative potential between the two branches of the Y, leaving the potential of the other branch floating. The cells in the floating channel remained stationary while the potential was applied to the other branch.

Mobilization and control of flow direction were also possible with red blood cells at both the double-T and Y-intersection geometries. Erythrocyte velocities were 0.058 ± 0.007 mm/s at 90 V/cm in an isotonic buffer solution. Experiments performed in a different buffer, containing 137 mM NaCl and 2.7 mM KCl, 4.2 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 , and 1.1 mM dextrose gave higher velocities, e.g., 0.55 ± 0.09 mm/s at 140 V/cm.

Surface Coatings

Cells have a tendency to adhere to capillary walls. The yeast and *E. coli* cells in initial experiments performed with cell counts of $4 \times 10^8/\text{mL}$ and $13 \times 10^8/\text{mL}$ for yeast and *E. coli* cells, respectively, adhered to the capillary walls. Reduction in
5 concentrations to $1 \times 10^8/\text{mL}$ and $3 \times 10^8/\text{mL}$ for yeast and *E. coli* cells, substantially eliminated the problem. It was found that devices with a $15 \mu\text{m} \times 55 \mu\text{m}$ cross section exhibited less cell sticking for yeast and erythrocytes than devices with channels about $30 \mu\text{m} \times 70 \mu\text{m}$ in cross section. However, in all devices there was some settling of the yeast and red blood cells in the solvent reservoirs contacting the
10 channels. This meant that the cell concentration inside the chip channels was not always equal to that introduced into the reservoirs. Nevertheless, experiments could be performed for several hours before it was necessary to change the sample reservoir cell suspensions.

Treating the capillary walls with a commercial trichlorohexadecylsilane agent
15 to make the walls hydrophobic also reduced problems with the cell adhesion. This coating substantially reduced electroosmotic flow, so that red blood cells in isotonic solution showed net migration in the direction of the anode. Nevertheless, sufficient residual charge remained on the channel walls that, in the distilled water used with the yeast or *E. coli* cells, electroosmotic flow still resulted in net migration toward the
20 cathode.

Lysis of Canine Erythrocytes

To illustrate reaction of the cells after their transport to a certain location, a simple experiment involving the lysis of erythrocytes (or hemolysis) by a detergent
25 was performed. This reaction occurs after mixing a stream of cells at an intersection with another stream containing a lysing agent. It was found that the anionic detergent, sodium dodecyl sulfate (SDS), lysed cells sufficiently rapidly that the lysing process could be followed in a flowing stream within the chip. While SDS will

modify the electroosmotic flow rate, the direction of solvent flow will continue to be toward the cathode, so flow remained well behaved.

Two slightly different experiments were performed to study erythrocyte lysis. In one study, a photomultiplier tube ("PMT") detector was located downstream of a mixing point, near the double-T of the PCRD2 device shown in Figure 5B. A potential of 400 V was applied between S and SW reservoirs to direct a stream of cells. A potential of 400 V was then periodically connected to reservoir B, which contained a lysing agent consisting of 3 mM SDS solution in deionized water. These potentials gave a field strength of 380 V/cm between the intersection and the detection point when only the cell reservoir was connected. A field strength of 520 V/cm was present when both cell and SDS channels were connected. The cell lysis reaction was followed by measuring the change in scattered light from the cells (using an epiluminescent microscope) at a location 2.5 mm downstream of the mixing point. The signal dropped as SDS was mixed with the flowing cell stream and then returned to its original range once the SDS flow was stopped. The signal fluctuates in the presence of the cells due to the discrete variation in the number of the cells in the detection volume as a function of time. The experiment shows the ability of electrokinetic valving action to control delivery of the lysing reagent on demand, as well as the on-chip chemical processing of cells.

In a second experiment, cells were introduced in the buffer reservoir, with 3mM SDS present in the sample reservoir and both reservoirs were at 150 V. The sample waste reservoir was at ground, causing a steady stream of cells and SDS to mix in the double-T region and migrate around the corner toward sample waste (130 V/cm about 0.058 ± 0.007 mm/s for the cells). Figure 10A shows cells entering from the left, with SDS mixing in from above. In Figure 10B, the two cells marked 1 and 4 have begun to react with SDS and lyse. The SDS has not diffused across the whole channel, so cells 2 and 3 along the wall are still intact by visual inspection. Figure 10C shows that the remains of cell 1 have been transported out of view, while

the other three marked cells are also now lysed. A total time of 0.3 s elapsed for these three frames.

Example III - Calcium Influx Assay

5 Calcium influx is involved in major cell functions and responses of lymphocytes. In this assay, the calcium ion influx into the lymphocytes is induced by a calcium releasing agent, calcimycin (A23187, calcium ionophore). The rise in the concentration of free intracellular calcium ions results in an increase in fluorescent signal from cells pretreated with a fluorescing agent. The kinetic results of calcium
10 influx may be studied with the stop flow method in the microfluidic device.

There are several steps in the study of the process of calcium influx. First, an activator, calcimycin (A23187 calcium ionophore) and the calcium ion complex must adsorb on the cell surface. Second, the complex has to diffuse through the cell membrane. Third, once inside the cytosol, dissociation between the A23187 and
15 calcium ion has to take place. Finally, a fluorescing agent, Fluo-3 AM, complexes with the released calcium and produces emitted light that can be detected (fluorescence).

Reagents and Chemicals

20 RPMI 1640 without phenol red tissue culture medium and Delbecco's phosphate buffered saline ("PBS") were obtained from Gibco BRL (Burlington, Ontario, Canada). The long-wavelength cytosolic calcium indicator Fluo-3 AM, nonionic detergent PLURONIC F-127, and calcium ionophore A23187 free acid (calcimycin) were obtained from Molecular Probes (Eugene, Oregon). Human serum
25 albumin (HSA) was obtained from Bayer Corp. (Kankakee, Illinois). The LYMPHOLYTE-poly was obtained from Cedarlane (Hornby, Ontario, Canada). All other chemicals including verapamil, mefenamic acid, fetal calf serum (FCS), D-(+)-glucose, N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES), and

ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (Oakville, Ontario, Canada).

Preparation and Labelling of Human Lymphocytes

5 For measurement of calcium influx, a calcium-specific fluorophore, Fluo-3 AM (Molecular Probes), was preloaded into purified human lymphocytes as acetoxymethyl (AM) ester.

Human lymphocytes from venous blood of healthy donors were isolated by LYMPHOLYTE-poly gradient centrifugation as described in Boyum (22). After
10 isolation of mononuclear cells, residual platelets were removed from the cell suspension by resuspending the cell pellet in PBS / 3mM EDTA / 1% HSA defined and centrifuged at 800 rpm for 12 minutes. Cells were washed three times with the above procedure. The purified lymphocyte pellet was resuspended in 5.0 ml HEPES buffer without calcium (5mM KCl, 145 mM NaCl, 1 mM Na₂HPO₄, 0.5 mM glucose,
15 10 mM HEPES, pH 7.4).

A 10 µl sample of Fluo-3 AM solution was diluted into 5.0 ml HEPES buffer without calcium. The 5.0 ml lymphocyte solution was mixed with the 5.0 ml Fluo-3 AM solution to make a 2 µM lymphocyte-Fluo-3 AM labeling solution. The lymphocytes were incubated for 60 minutes at room temperature on a hematology
20 mixer. This Fluo-3 AM labeling procedure was followed as described by Hagar et al.,(21).

After lymphocyte labelling, the cells were washed three times with HEPES buffer without calcium. The labeled lymphocytes were suspended at 1X10⁷ cells/ml in RPMI 1640 without phenol red media and kept at 22 °C until needed.

25

Determination of Mixing Time

To determine the time of mixing at point B in Figure 7A, a dye, Bengal Rose (5mM) was introduced into the activator channel (#4) of PCRD1 (Figure 8) prepared by the method set forth in Example 1. The flow is laminar, and at the flow velocity

there is no diffusion of dye into the buffer during transit time. After stop-flow, Figure 7B shows the extent of diffusion of dye after 0.2 seconds into the buffer. No gradient was visible to the CCD detector after about 0.4 seconds. Simple diffusion estimates indicate that uniform mixing should take less than 1 second. This allows for studies of the kinetics of individual cell processes that are slower than 1 second.

Preparation of the Microfluidic System

5% fetal calf serum ("FCS") in RPMI 1640 without phenol red media (Gibco BRL, Burlington, Ontario, Canada) was either contained in the cell media and/or flushed through the microfluidic system for 30 minutes before use. The FCS coated channels significantly reduced electroosmotic flow. Thus a hydrodynamic flow system, using negative pressure applied by syringe suction, was used for the stop flow method.

15 Use of the Microfluidic System

Figures 8 and 9 show the layout of the post column reaction manufactured by the method set forth in Example 1.

With reference to Figure 8, buffer was added to channels #1 and #2, lymphocytes, prepared as described above, were added to channel #3, and activator A23187 was added to channel #4.

Solvent flow was driven by suction (negative pressure) applied to the waste outlet with a manually operated glass syringe. The negative pressure draws solution from all four inlet reservoirs, with approximately proportionate amounts drawn from the activator reservoir as compared to the other three reservoirs combined.

25 A fluorescence microscope equipped with a 25X objective and a CCD camera was positioned over the detection zone, point "B" in Figure 8, allowed the observation of cells as they passed the detection zone. The device was operated in a stop flow mode in which flow was stopped when a single cell entered the detection zone for observation. Stopping the flow allowed the two unmixed zones at the

intersection to diffuse into each other, i.e. the main flow path and the activator flow path, beginning cell reaction with the added activator reagent.

After 4 seconds at the mixing point, the lymphocyte showed fluorescence caused by calcium influx upon activation with A23187. The increase in emitted light from nonactivated to activated calcium channels in cells has earlier been reported to be between 2 and 40 times depending on cell type and method. The increase in emitted fluorescence observed was typically on the order of 5 to 10 fold. A kinetic plot of the calcium flux on a human lymphocyte is shown in Figure 12.

The solution in the inhibitor reservoir was replaced with buffer from noninhibited control experiments. Alternatively, for greater flexibility of the process, a second syringe can be placed on the activator reservoir (channel #4) or on the buffer reservoir (channel #1), in order to prevent flow from those channels while the cells are delivered to point "B". This allows for ready implementation of control experiments.

The dual syringe control technique was investigated using the Rose Bengal dye in point "B" on the chip. With reference to Figure 8, buffer was added to flow paths #1, #2, and #3 and Rose Bengal dye was added to flow path #4. The syringe was connected to the "waste" and flow paths number #4. By changing the back pressure or flow rate, it was possible to control the flow from 0% dye to 100% dye. However, a small back-flow into the "stopped flow" channel was needed to assure "stopped flow" in the channel.

Example IV - Calcium Influx Assay with Lymphocyte Inhibitor

The microfluidic device and human lymphocytes were prepared as described in Example III.

With reference to Figure 8, 100 μ M of verapamil, an inhibitor, was introduced into channel #1; buffer was introduced into channel #2; leukocytes were introduced into channel #3; and calcium activator A23187 was introduced into channel #4.

The device was run as described in Example III above. Verapamil was introduced to the lymphocytes at point "A" intersection of the device (Figure 8). The lymphocytes were incubated with verapamil for four minutes while transported through the main channel to the mixing point "B". Observation of the verapamil treated lymphocyte showed no fluorescence. Thus, verapamil caused a significant reduction of activation in calcium flux on the human lymphocytes.

This example was completed on twenty individual lymphocytes and each showed comparable results. The verapamil inhibition was time dependent, as lymphocytes incubated for 30 seconds with verapamil in the mixing channel showed no inhibition.

Example V - Oxidative Burst

The production of superoxide anion O_2^- can be measured using the oxidant-sensitive fluorescent dye, dihydrorhodamine 123 (Molecular Probes). Granulocytes are incubated in the presence of 2 μ M dihydrorhodamine 123 for 20 minutes at 37°C. Labelled cells are then washed and resuspended in PBS. These cells are then analyzed for the induction of the oxidative burst pathway using the microfluidic device as described in Example III. Using stop-flow, single granulocytes are mixed with an activator such as fMLP and the induction of fluorescence is monitored. Similar types of fluorescence based assays may be used for agonists that induce intracellular pH changes or to study programmed cell death.

Example VI - Weir Device

Mice lymphocytes (5×10^7) were flushed into the chip with PBS. Two weir devices were used in the microfluidic device. The distance between the weirs in the first device was 1 μ m and in the second device it was 3 μ m. Flow was induced by pressure applied by a syringe.

In both devices the lymphocytes migrated through the weir. When the cells entered the weir they occupied all areas where liquid passes, thereby forming an

effective plug that reduced the fluid flow. Higher pressure was then needed to start the cells moving again. This resulted in the cells migrating over the top of the weir.

Figures 4A and B show a schematic of a weir-based device for physical trapping of cells fabricated using a two-mask process in glass. The kinetics of calcium uptake and release from a single cell can be followed within these structures.

Example VII - Selectin Binding to Select Channels of a Microfluidic Device

This example shows that selectins can be bound to specific flow paths in the device of the present invention.

The microfluidic flow paths, as shown in Figure 8 (PCRD1) and Figure 5B (PCRD2) were first vacuumed to remove dust or debris particles that may lodge in the flow paths and cause blockage. Filtered Dulbecco's phosphate buffered saline (PBS) (Gibco) was added to the flow paths and flushed through with a vacuum for 30 minutes at room temperature. After flushing the microchip, the flow paths were checked for air bubbles. Air bubbles were removed from the flow paths with continuous vacuum.

In each experiment, the microfluidic device was incubated for 2 hours at 37°C during the flow of selectin through the flow paths. After the incubation period, the flow paths were flushed with PBS for 10 minutes under vacuum. 100 μ l of a 1:10 dilution of mouse monoclonal primary antibody (anti-P or E-selectin) was then added to bind to the previously bound selectin. This primary antibody was flushed through the flow paths with back pressure for 1.0 hour. After primary antibody incubation, the flow paths were flushed with PBS.

100 μ l of a 1:10 dilution of secondary antibody (Goat F(ab')₂ anti-mouse IgG (H+L)) labeled with fluorescein (Pierce) was then added to the flow paths. The secondary antibody was vacuumed through the flow paths for 15 to 30 minutes at 37°C. After the secondary antibody incubation, the flow paths channels were again flushed with PBS while visualizing the selectin bound flow path under a microscope.

Once the unbound fluorescent secondary antibody was washed away, the bound secondary antibody was visible on the channel walls indicating that the corresponding selectin was bound to the channel wall.

5 In the first experiment, purified selectins (P or E) was added to the waste outlet of PCRD1 (Figure 8); the syringe was placed on inlet #4 and the fluid was drawn to inlet #4. Primary antibody was added to the waste outlet and the syringe was placed on inlet #2, and the antibody was drawn to inlet #2. Finally secondary antibody was added to inlets #1, #2, #3, and #4 and the syringe was placed on the waste outlet and the secondary antibody was drawn to the waste outlet. Fluorescence
10 was seen in the waste channel only.

In the second experiment, membrane extracts containing selectins were added to flow paths #1 and #2 and fetal calf serum was added to flow path #3. A syringe was placed on the waste flow path outlet and the selectins and fetal calf serum drawn to the waste outlet. Primary antibody was added to flow paths #1, #2, #3, and #4 and
15 the syringe was placed on the waste flow path outlet and drawn. Secondary antibody was also added to flow paths #1, #2, #3, and #4 and the syringe was placed on the waste flow path outlet and drawn. Fluorescence was seen in paths #1 and #2 but not in paths #3 or #4.

This illustrates that selectins can be added to other flow paths and the area of
20 selectin binding to specific sites on the flow paths controlled.

Example VIII - Cell Rolling

Leukocytes move into tissues under three mechanisms. First, naive leukocytes have a homing response or migration via high endothelial venules into secondary
25 lymphoid tissue. Second, stimulated leukocytes and/or memory cells display tissue-restricted migration to sites such a mucosal epithelium or skin. Finally, leukocytes as well as neutrophils and monocytes, transmigrate into inflamed tissues in response to localized stimuli. The basic molecular mechanisms of the inflammatory response has been characterized and comprises a cascade of events brought about by the sequential

binding of different adhesion receptors. The first step in this adhesion cascade is the reversible binding mediated by selectins. Selectins cause the leukocytes to roll along the inflamed endothelium. During the next phase, a leukocyte activation event mediated by cytokines induces leukocytes to flatten on the endothelium, resulting in transmigration into the tissue. Transmigration depends upon the integrin-ligand binding. It is now recognized that the basic steps of this adhesion cascade and the receptors involved in the process are also used in the trafficking of naive and memory leukocytes.

Conventional rolling cell methods require up to 50 ml of solution per test, or the dosage of an animal in the range of 100mg/kg. In contrast, a typical drug dosage in the range of 100 μ g/ml of blood would lead to only 1-20 μ g of proto-drug required for tests on hundreds of thousands of cells using this system.

Flow conditions in the cell rolling chamber mimic physiological conditions. The requirements are an average flow rate, v_{mean} , of 1-4 mm/s, with a shear rate, $\tau = 8v_{\text{mean}}/d$ (s^{-1} , where d is capillary diameter), of about 800 1/s, giving a relationship between velocity and diameter of $v_{\text{mean}} = 100d$. A velocity of 1-5 mm/s is needed.

Purified selectins are adsorbed in defined regions within the microfluidic flow path as described above. The dimensions for the device to evaluate the effect of a drug on cell rolling is a depth of 10 μ m, a width of 30-300 μ m, and a 3 cm length, giving volumes of 10-100 nL. A linear flow rate of 2 mm/s gives a flow rate of 0.6 to 6 nL/s. The volume inside the flow paths is insignificant as compared to the 10-200 μ L in the contact reservoir or the syringe pump.

Modification of the above-described modes of carrying out various embodiments of this invention will be apparent to those skilled in the art following the teachings of this invention as set forth herein. The examples described above are not limiting, but are merely exemplary of this invention, the scope of which is defined by the following claims.

We claim:

- 1 1. A microfluidic device comprising:
2 a main flow path comprising a detection zone and an outlet; and
3 at least two inlet flow paths wherein the inlet flow paths intersect and merge
4 into the main flow path at or upstream of the detection zone at an upstream angle of
5 less than 90°.
- 1 2. The microfluidic device of Claim 1 further comprising two inlet flow
2 paths.
- 1 3. The microfluidic device of Claim 1 further comprising three inlet flow
2 paths.
- 1 4. The microfluidic device of Claim 3 wherein the main flow path has at
2 least one detection zone at or downstream of each intersection of each inlet flow path
3 with the main flow path.
- 1 5. The microfluidic device of Claim 1 wherein the main flow path is from
2 about 0.1 μm deep by 0.1 μm wide to about 1 mm deep by 2 mm wide.
- 1 6. The microfluidic device of Claim 1 wherein the first inlet flow path is
2 from about 0.1 μm deep by 0.1 μm wide to about 1 mm deep by 2 mm wide.
- 1 7. The microfluidic device of Claim 1 further comprising means for
2 applying a flow inducing force.
- 1 8. The microfluidic device of Claim 6 wherein the flow inducing force is
2 electricity.

1 9. The microfluidic device of Claim 6 wherein the flow inducing force is
2 negative or positive fluid pressure.

1 10. The microfluidic device of Claim 9 wherein positive or negative
2 pressure is applied to the outlet.

1 11. The microfluidic device of Claim 1 wherein the device further
2 comprises cells in at least one of the inlet flow paths and the main flow path.

1 12. The microfluidic device of Claim 1 wherein the device further
2 comprises leukocytes, a calcium dye and a candidate compound in the main flow path.

1 13. An observation device comprising a plurality of microfluidic devices of
2 Claim 1 sharing a common detection zone.

1 14. The observation device of Claim 13, wherein the main flow paths of
2 the microfluidic devices are substantially parallel at the common detection zone.

1 15. An observation device comprising a plurality of microfluidic devices of
2 Claim 1 wherein the main flow paths of the microfluidic devices are substantially
3 parallel at their detection zones.

1 16. A method of observing the effect of a candidate compound on cells in a
2 microfluidic device comprising:

3 (a) providing a microfluidic device comprising a main flow path comprising a
4 detection zone, and an outlet and at least two inlet flow paths intersecting and merging
5 with the main flow path at or upstream of the detection zone;

6 (b) applying at least one cell to a first inlet flow path and the candidate
7 compound to a second inlet flow path;

8 (c) inducing flow of the cells and the candidate compound toward the outlet;
9 (d) allowing the cells to mix with the candidate compound at the intersection of
10 the second inlet flow path and the main flow path; and
11 (f) observing the effect of the candidate compound on the cells in the detection
12 zone.

1 17. The method of Claim 16 wherein the microfluidic device has three inlet
2 flow paths and a second candidate compound is added to the third inlet flow path.

1 18. The method of Claim 16 further comprising stopping the flow of the
2 cells while the cells are in the detection zone.

1 19. The method of Claim 17 further comprising observing the cells in each
2 of a number of detection zones wherein the main flow path comprises a plurality of
3 detection zones, wherein each detection zone is at or downstream of each intersection
4 of each inlet flow path with the main flow path.

1 20. The method of Claim 16 wherein the candidate compound being studied
2 is a cell activator and the cell is a lymphocyte.

1 21. The method of Claim 17 wherein cells are added to a first inlet flow
2 path, cell activator is added to a second inlet flow path, and a candidate compound is
3 added to a third inlet flow path.

1 22. The method of Claim 21 wherein the candidate compound being studied
2 is an inhibitor, and the cells are lymphocytes.

1 23. The method of Claim 16 wherein the flow paths are coated with a
2 substance selected from the group consisting of proteins, glycoproteins, phospholipids,
3 hydrophilic polymers and hydrophobic polymers.

1 24. The method of Claim 23 wherein the flow path is coated with protein.

1 25. The method of Claim 23 wherein the flow is induced by an electric
2 force.

1 26. The method of Claim 24 wherein the flow is induced by positive or
2 negative fluid pressure.

1 27. A method for studying calcium influx in a lymphocyte comprising:

2 (a) providing a microfluidic device comprising a main flow path having a
3 detection zone, at least two inlet flow paths sequentially intersecting with the main
4 flow path upstream of the detection zone and an outlet downstream from the detection
5 zone;

6 (b) applying lymphocytes to a first inlet flow path and an activator to a second
7 inlet flow path;

8 (c) inducing flow of the lymphocytes and the activator toward the outlet;

9 (d) allowing the lymphocytes to mix with the activator at the intersection of the
10 second inlet flow path and the main flow path; and

11 (e) observing the effect of the activator on the lymphocytes in the detection
12 zone.

1 28. The method of Claim 27 wherein the microfluidic device comprises
2 three inlet flow paths further comprising adding a candidate compound to a third inlet
3 flow path; and observing the effect of the candidate compound on the lymphocytes in
4 the detection zone.

- 1 29. A method for studying leukocyte rolling comprising:
- 2 (a) providing a microfluidic device comprising a main flow path comprising a
- 3 detection zone and an outlet and at least two inlet flow paths sequentially intersecting
- 4 and merging with the main flow path at or upstream of the detection zone and wherein
- 5 the walls of the main flow path in the detection zone have attached thereto cell
- 6 adhesion molecules;
- 7 (b) applying at least one leukocyte to a first inlet flow path;
- 8 (c) applying a candidate compound to a second inlet flow path;
- 9 (d) inducing flow of the cells and the compound into the main flow path;
- 10 (d) allowing the leukocytes, candidate compound and cell adhesion molecules
- 11 to interact; and
- 12 (e) observing the leukocyte rolling in the detection zone.

- 1 30. The method of Claim 29 wherein the device comprises three inlet flow
- 2 paths, further comprising adding an inhibitor to said third inlet flow path; mixing the
- 3 inhibitor, leukocytes, candidate compound and cell adhesion molecules and observing
- 4 the leukocyte rolling in the detection zone.

- 1 31. The method of Claim 30 further comprising stopping the flow of the
- 2 cells, candidate compound and inhibitor during the mixing step.

- 1 32. The device of Claim 1 further comprising variations in the cross-section
- 2 of the main flow path.

- 1 33. The device of Claim 32 wherein the variations create a cell trapping
- 2 zone.

- 1 34. The device of Claim 33 wherein said variations are weirs.

1
2
3
4
5

Abstract

This invention relates to a novel microfluidic device and methods of using this device to conduct *in vitro* studies on the reaction and effects of various compounds on cells. More particularly, it relates to a method for using stop flow in a microfluidic system to study the effect of compounds on individual cells.

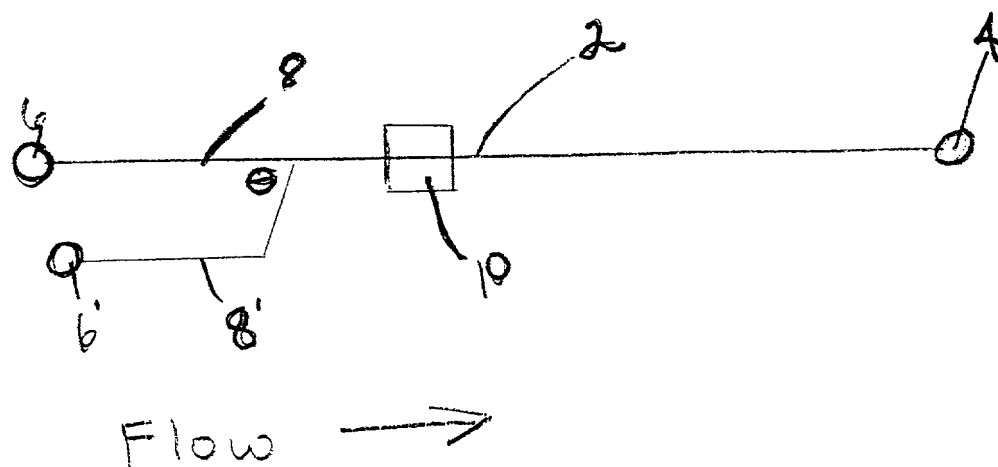


Figure 1

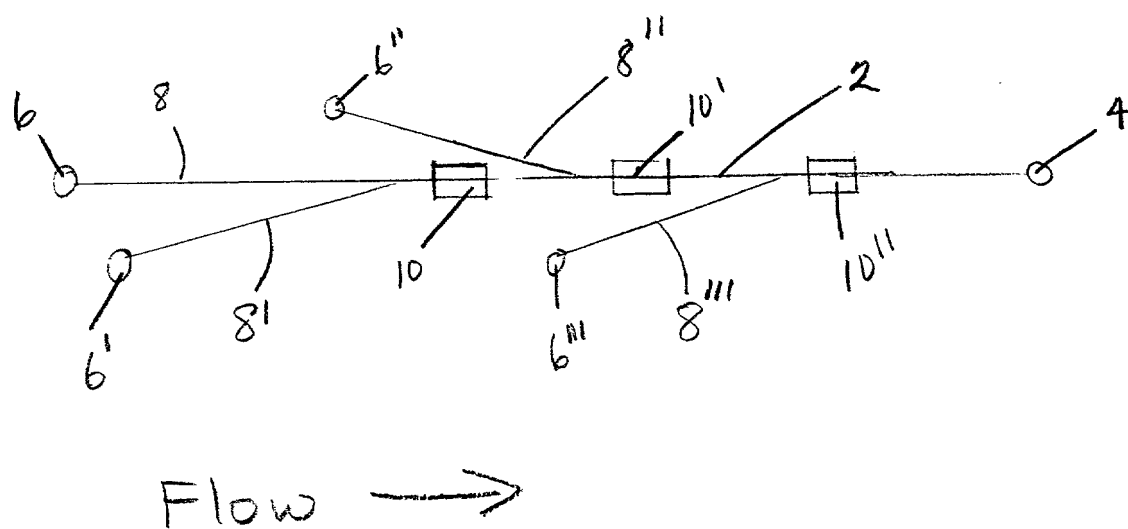


Figure 2

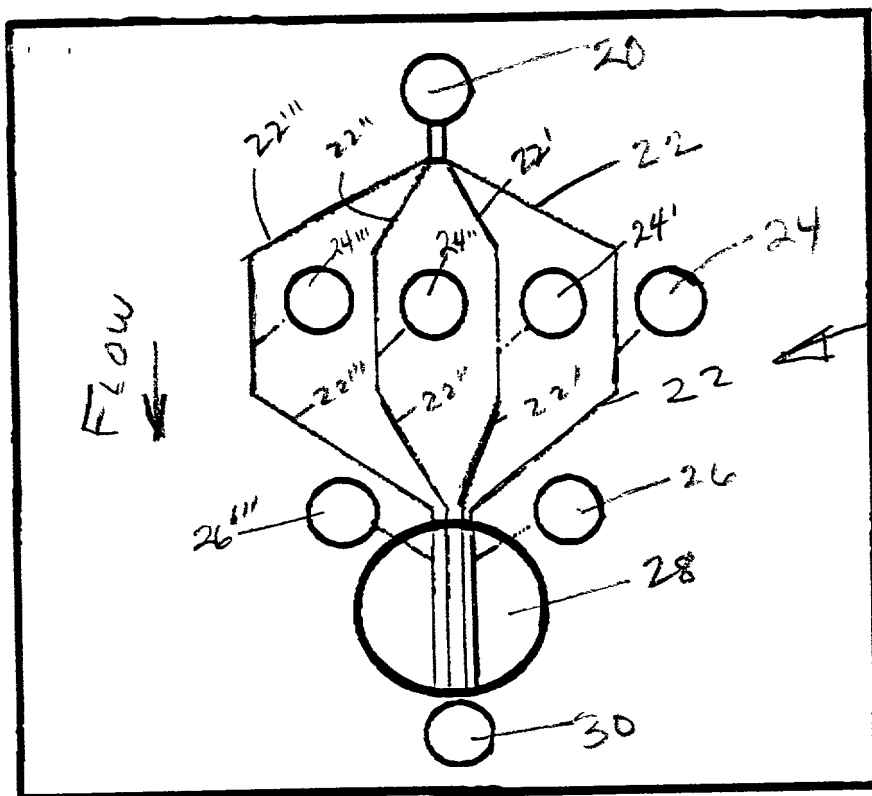


Figure 3A

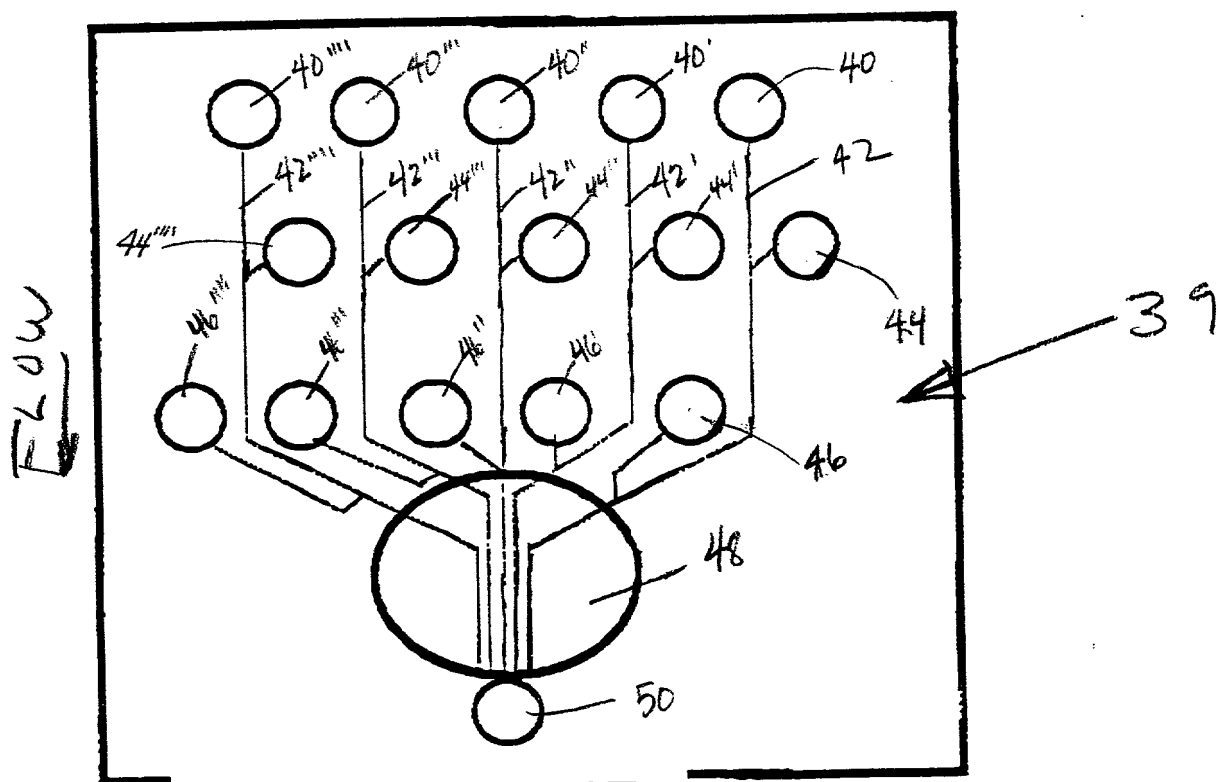


Figure 3B

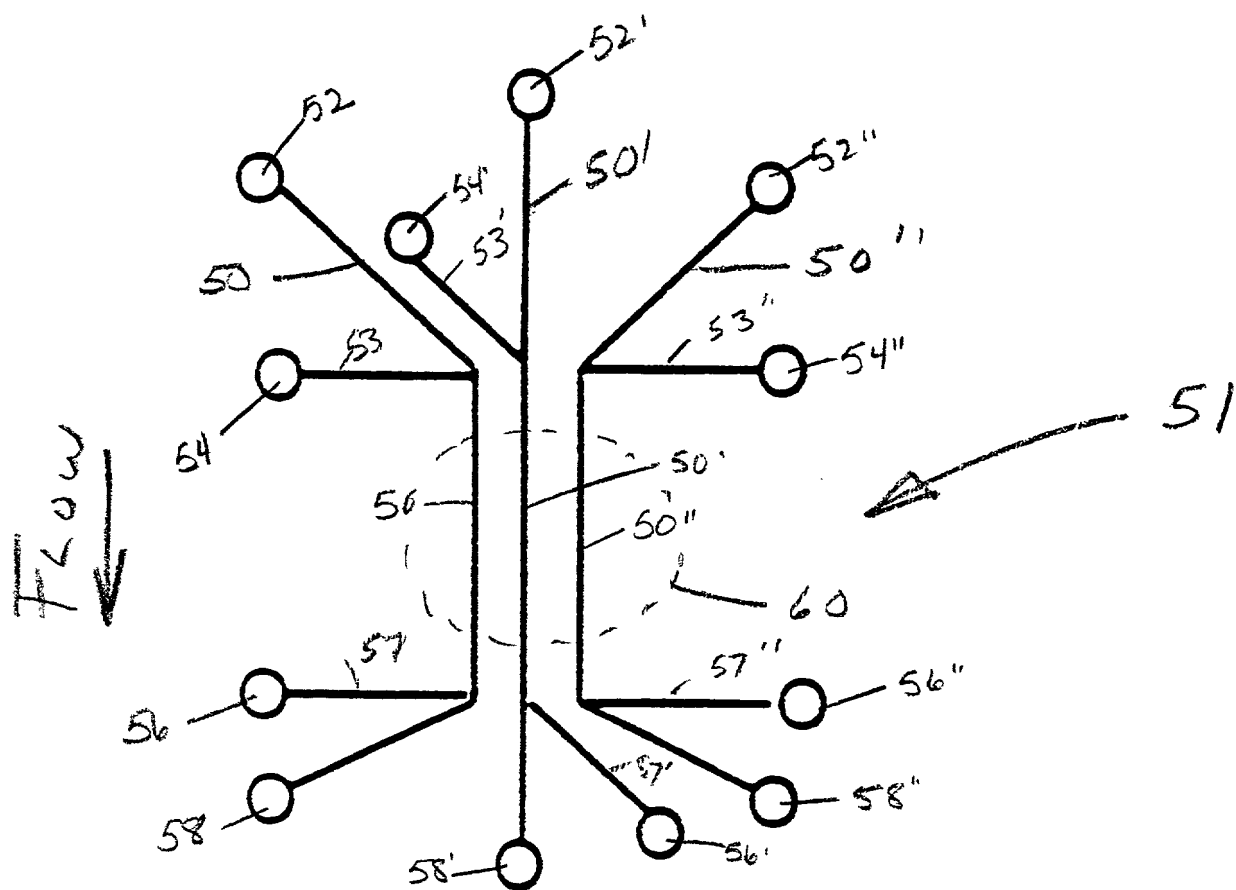


Figure 3C

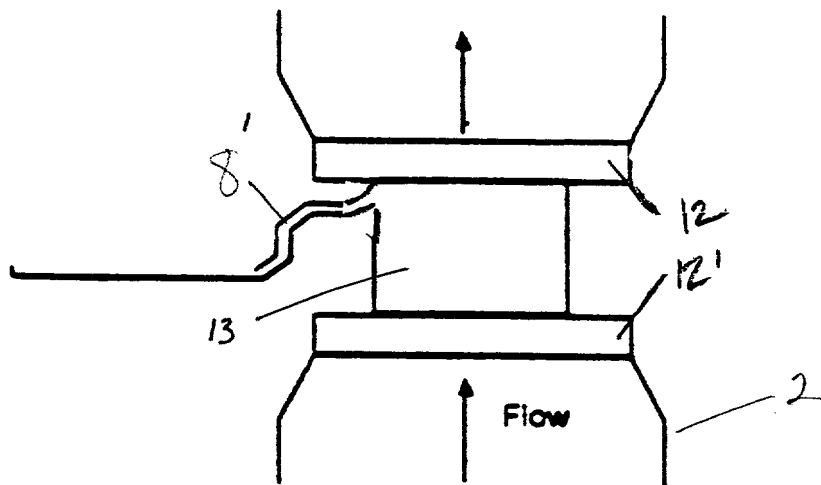


Figure 4A

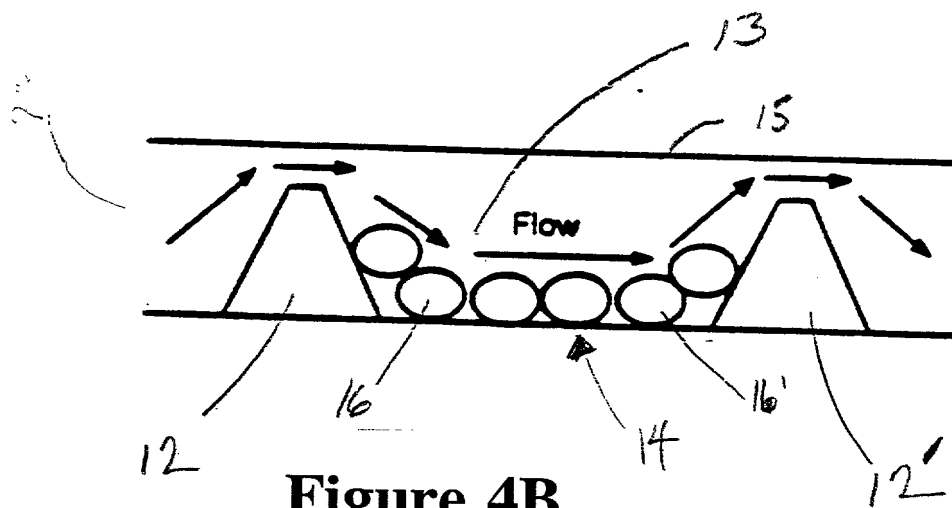


Figure 4B

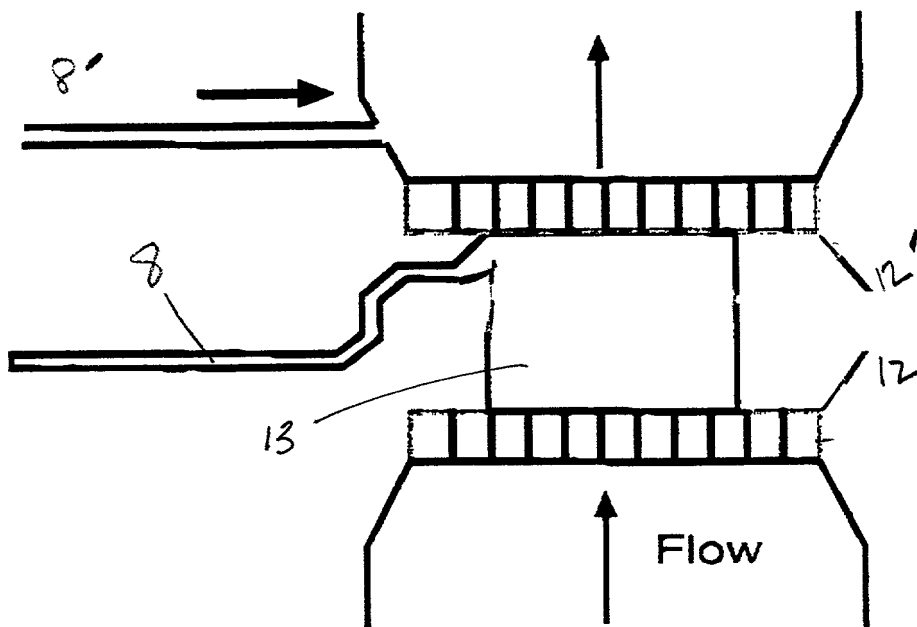


Figure 4C

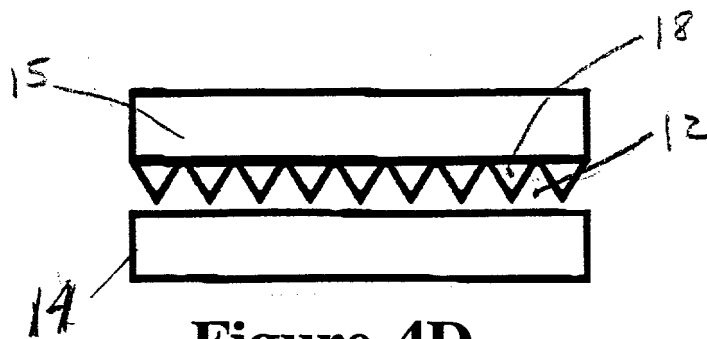


Figure 4D

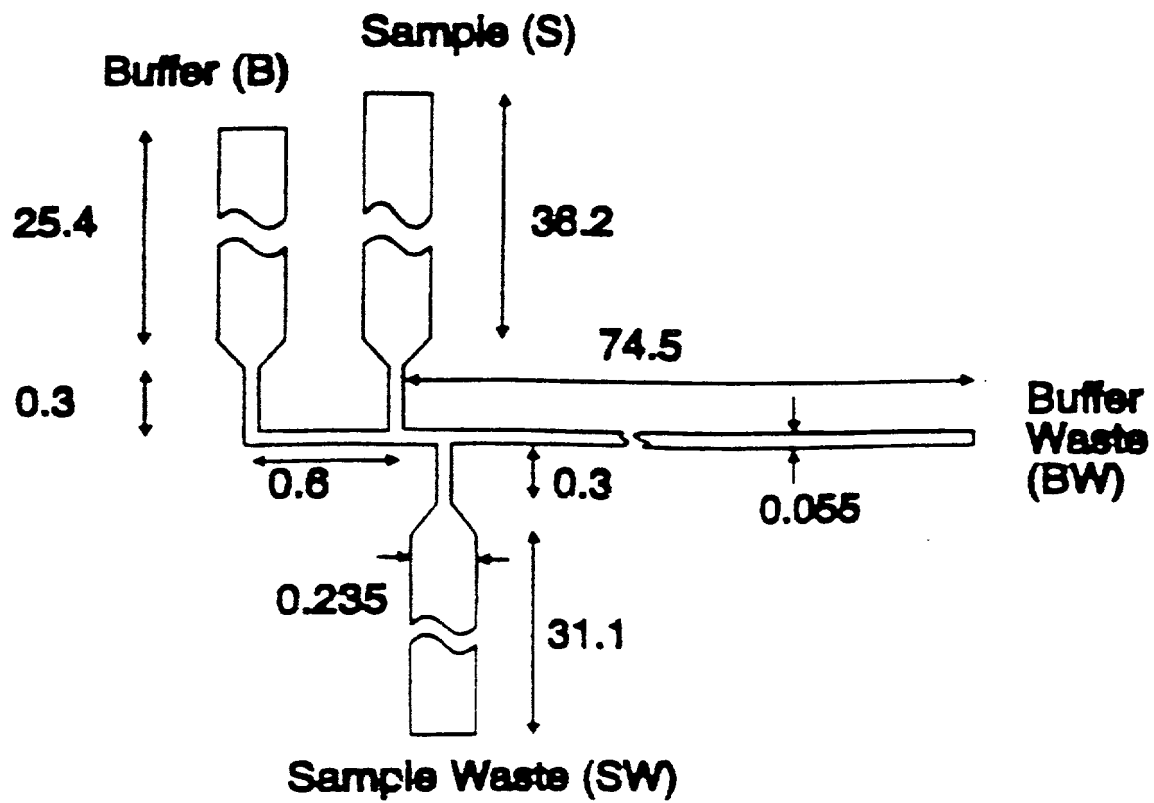


Figure 5A

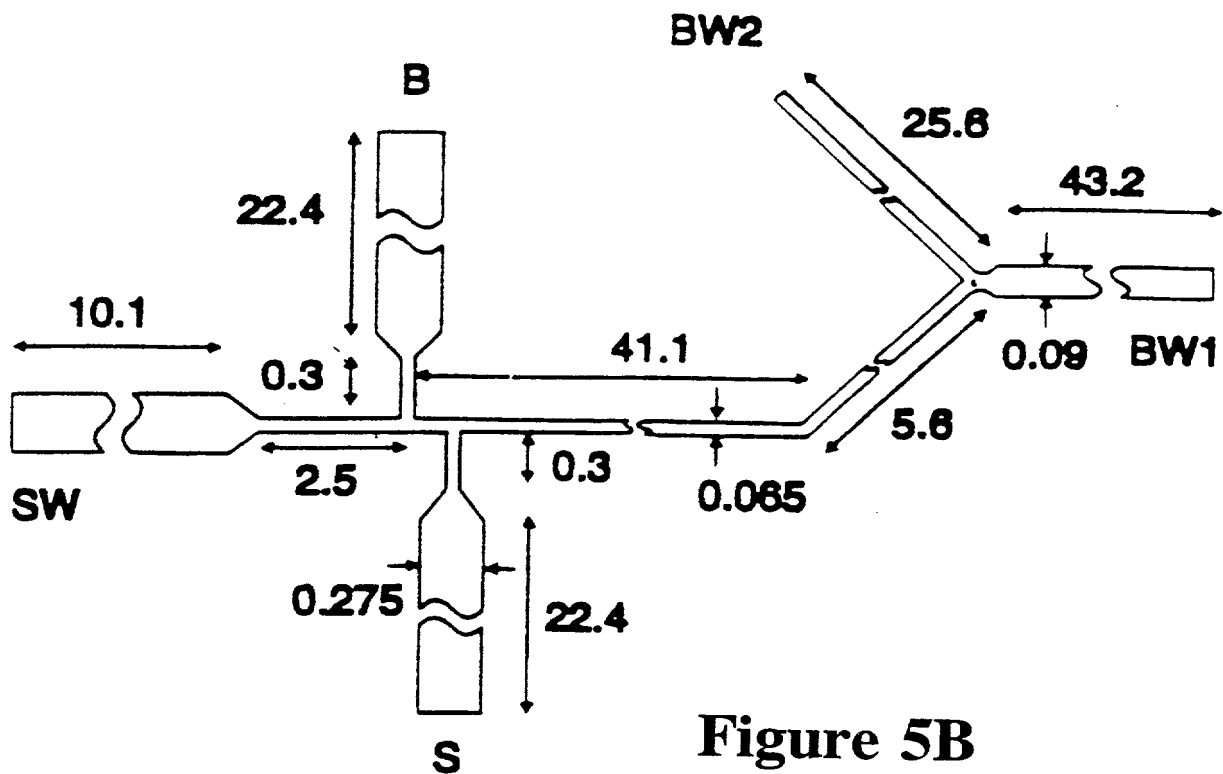


Figure 5B

0255007 05459
2005007 2005008

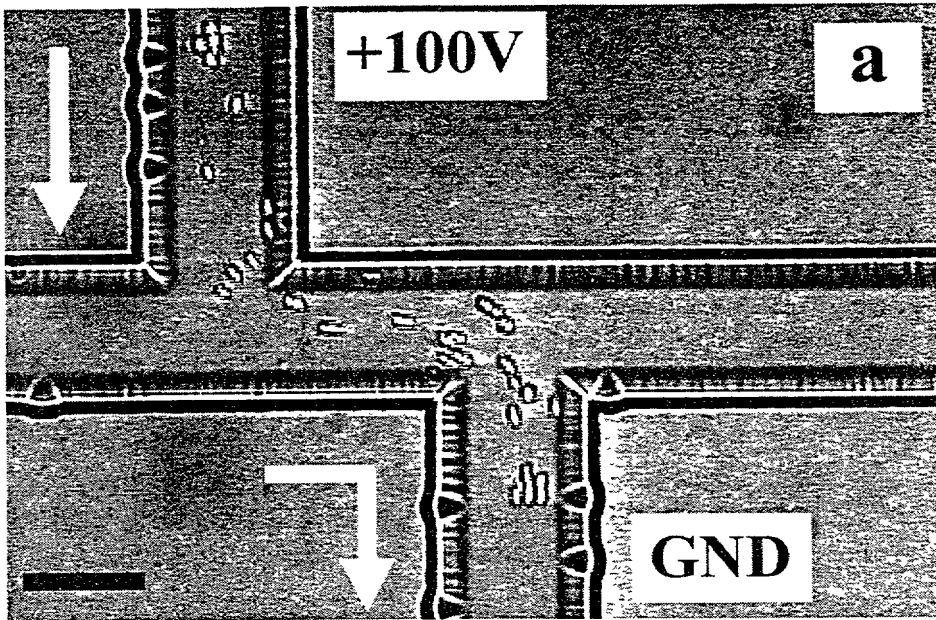


Figure 6A

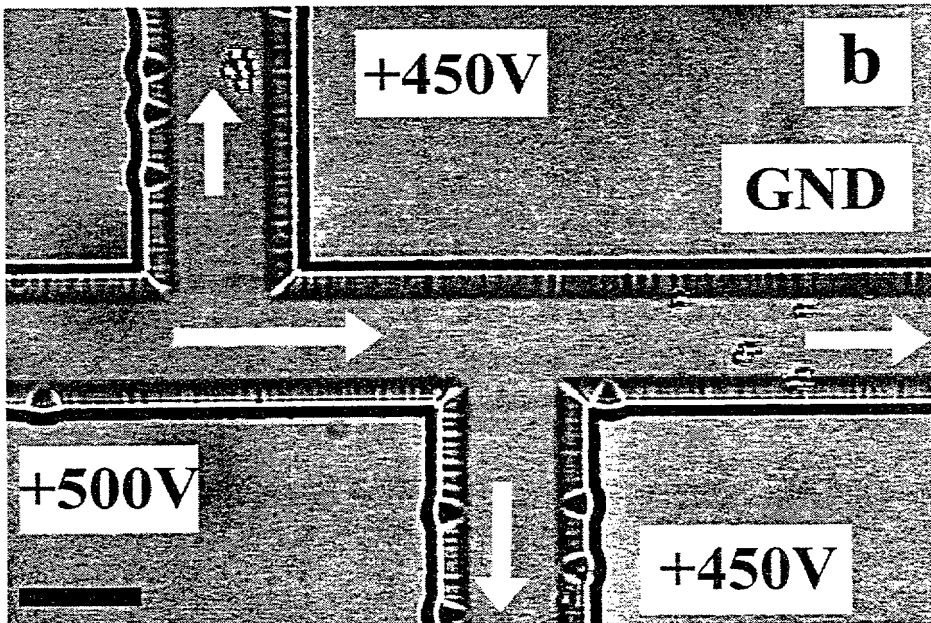


Figure 6B

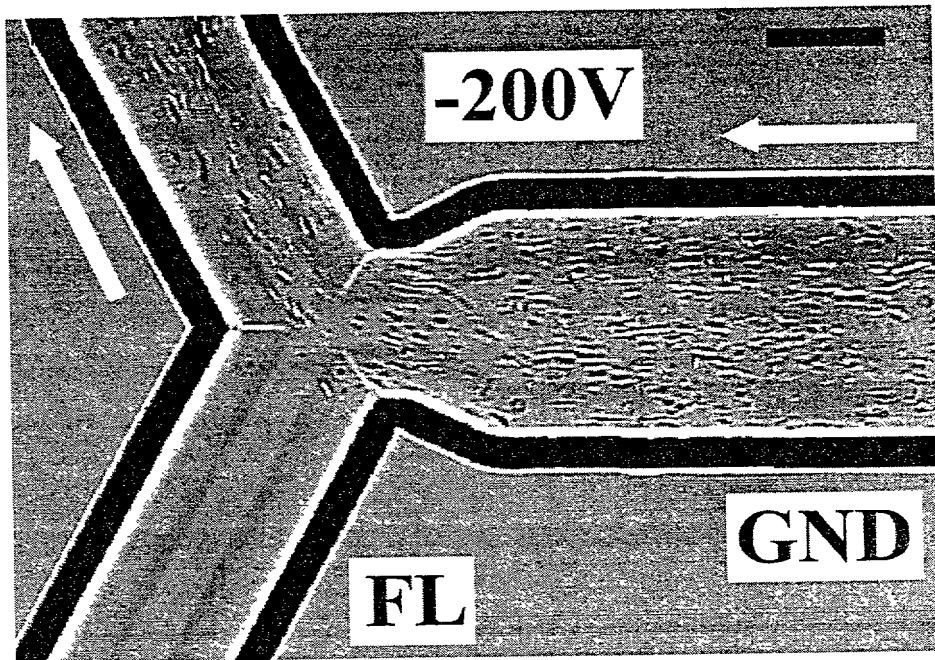


Figure 6C

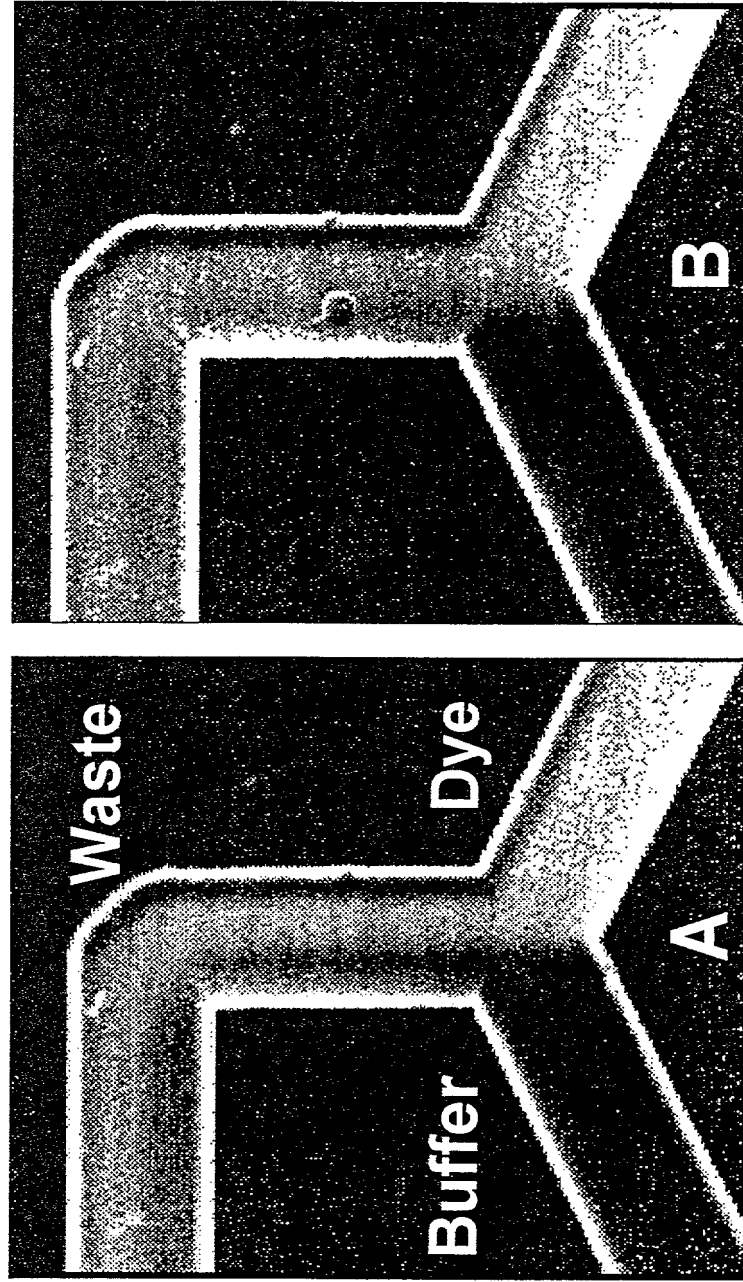


Figure 7

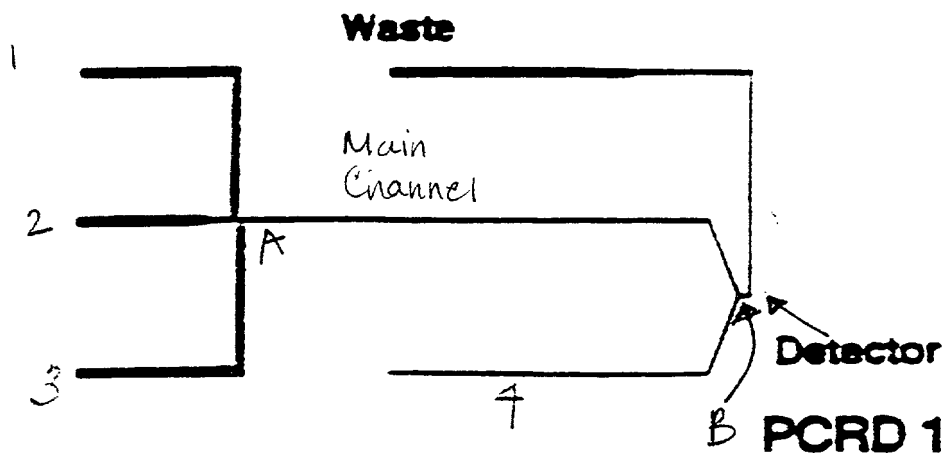


Figure 8

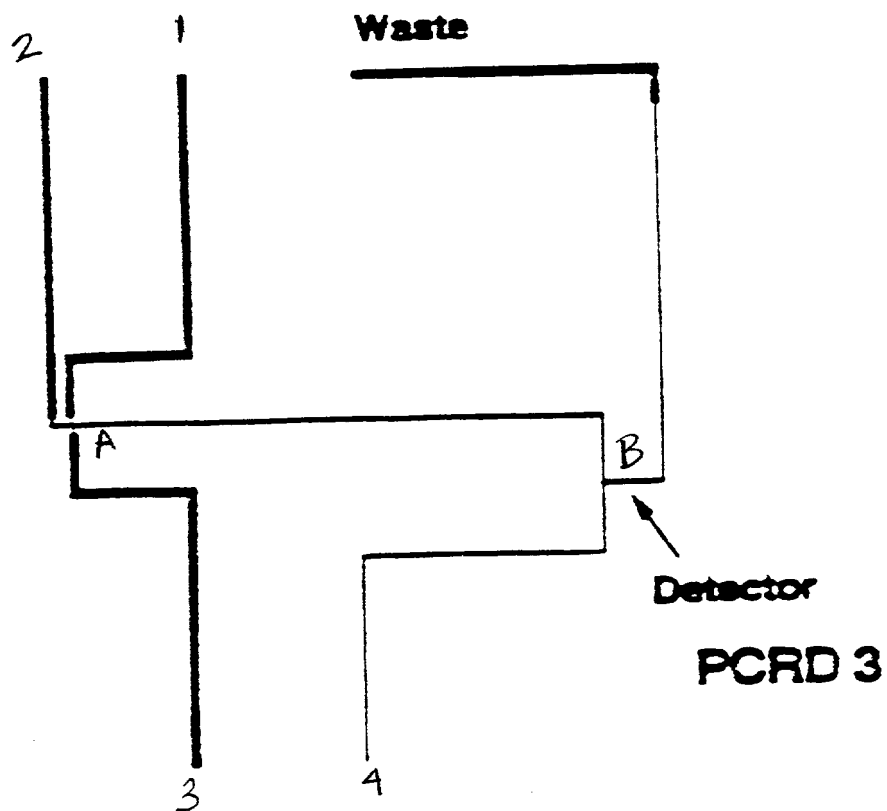


Figure 9

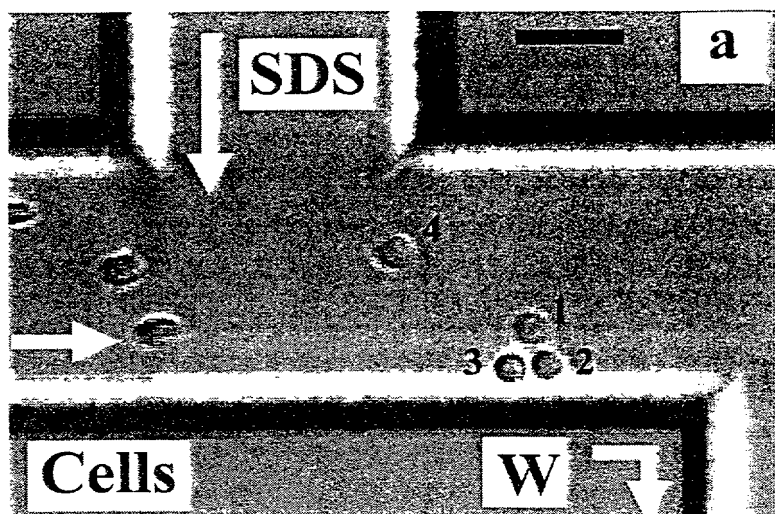


Figure 10A

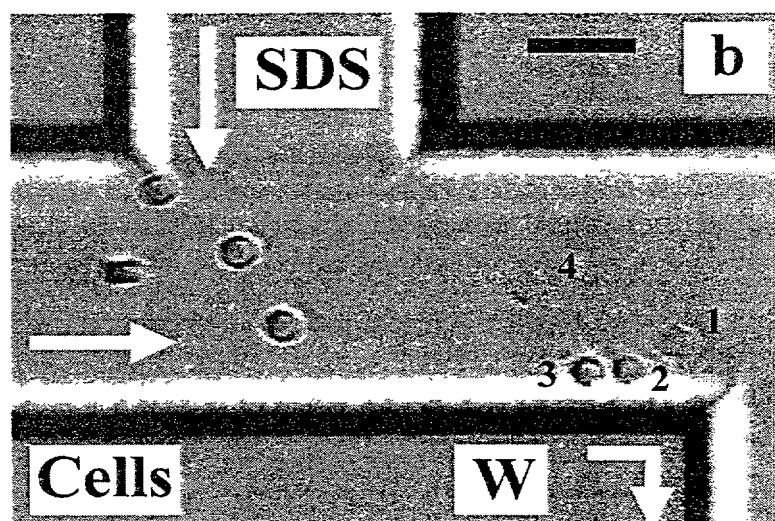


Figure 10B

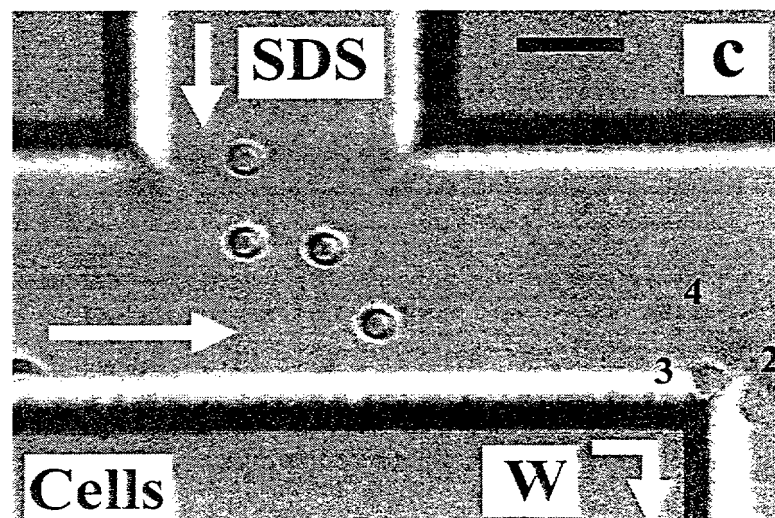


Figure 10C

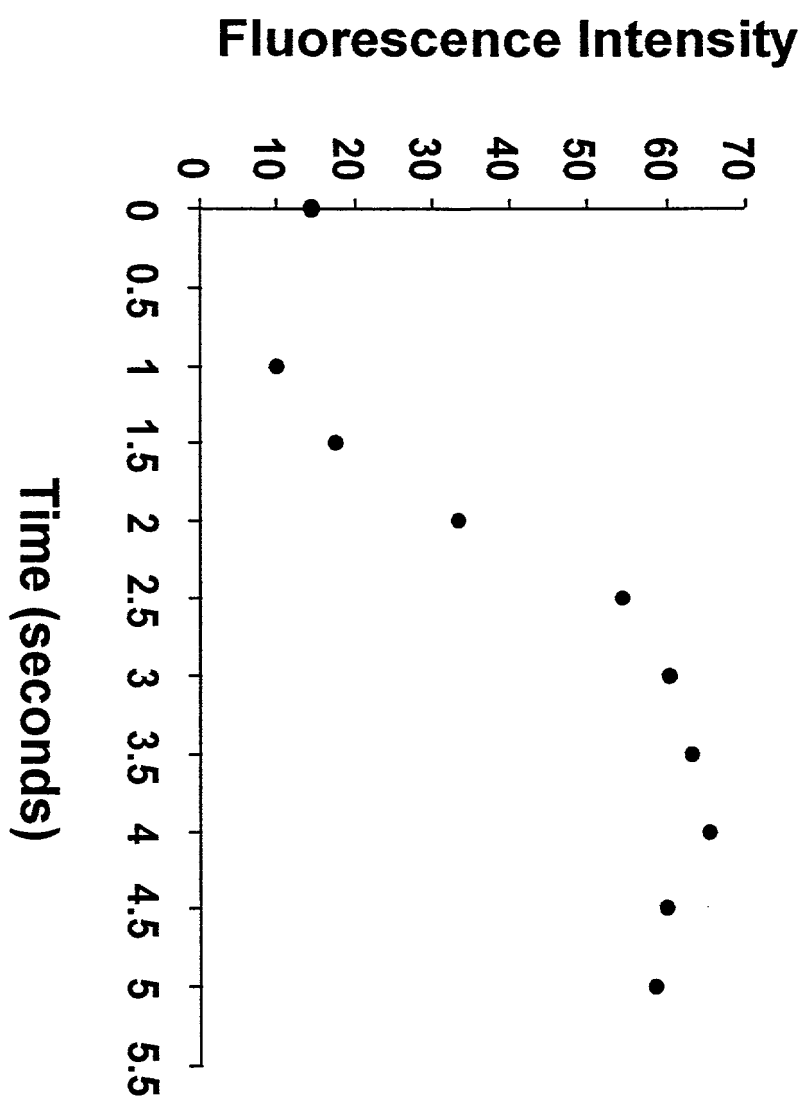


Figure 11

**COMBINED DECLARATION AND POWER OF ATTORNEY
FOR UTILITY PATENT APPLICATION**

Attorney's Docket No.

000475-205

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (if only one name is listed below) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (if more than one name is listed below) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED:

MICROFLUIDIC SYSTEM AND METHODS OF USE

the specification of which

(check one)

☒

is attached hereto;

☐

was filed on _____ as

Application No. _____

and was amended on _____;
(if applicable)

I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE;

I ACKNOWLEDGE THE DUTY TO DISCLOSE TO THE OFFICE ALL INFORMATION KNOWN TO ME TO BE MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS, Sec. 1.56 (as amended effective March 16, 1992);

I do not know and do not believe the said invention was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to said application; that said invention was not in public use or on sale in the United States of America more than one year prior to said application; that said invention has not been patented or made the subject of an inventor's certificate issued before the date of said application in any country foreign to the United States of America on any application filed by me or my legal representatives or assigns more than twelve months prior to said application;

I hereby claim foreign priority benefits under Title 35, United States Code Sec. 119 and/or Sec. 365 of any foreign application(s) for patent or inventor's certificate as indicated below and have also identified below any foreign application for patent or inventor's certificate on this invention having a filing date before that of the application(s) on which priority is claimed:

COMBINED DECLARATION AND POWER OF ATTORNEY			Attorney's Docket No. 000475-205																																																																									
COUNTRY/INTERNATIONAL	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED																																																																									
			YES_ NO_																																																																									
			YES_ NO_																																																																									
<p>I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">William L. Mathis</td> <td style="width: 10%;">17,337</td> <td style="width: 33%;">Ralph L. Freeland, Jr.</td> <td style="width: 10%;">16,110</td> <td style="width: 33%;">William C. Rowland</td> <td style="width: 10%;">30,888</td> </tr> <tr> <td>Peter H. Smolka</td> <td>15,913</td> <td>Robert G. Mukai</td> <td>28,531</td> <td>T. Gene Dillahunt</td> <td>25,423</td> </tr> <tr> <td>Robert S. Swecker</td> <td>19,885</td> <td>George A. Hovanec, Jr.</td> <td>28,223</td> <td>Anthony W. Shaw</td> <td>30,104</td> </tr> <tr> <td>Platon N. Mandros</td> <td>22,124</td> <td>James A. LaBarre</td> <td>28,632</td> <td>Patrick C. Keane</td> <td>32,858</td> </tr> <tr> <td>Benton S. Duffett, Jr.</td> <td>22,030</td> <td>E. Joseph Gess</td> <td>28,510</td> <td>Bruce J. Boggs, Jr.</td> <td>32,344</td> </tr> <tr> <td>Joseph R. Magnone</td> <td>24,239</td> <td>R. Danny Huntington</td> <td>27,903</td> <td>William H. Benz</td> <td>25,952</td> </tr> <tr> <td>Norman H. Stepno</td> <td>22,716</td> <td>Eric H. Weisblatt</td> <td>30,505</td> <td>Peter K. Skiff</td> <td>31,917</td> </tr> <tr> <td>Ronald L. Grudziecki</td> <td>24,970</td> <td>James W. Peterson</td> <td>26,057</td> <td>Richard J. McGrath</td> <td>29,195</td> </tr> <tr> <td>Frederick G. Michaud, Jr.</td> <td>26,003</td> <td>Teresa Stanek Rea</td> <td>30,427</td> <td>Matthew L. Schneider</td> <td>32,814</td> </tr> <tr> <td>Alan E. Kopecki</td> <td>25,813</td> <td>Robert E. Krebs</td> <td>25,885</td> <td>Michael G. Savage</td> <td>32,596</td> </tr> <tr> <td>Regis E. Slutter</td> <td>26,999</td> <td>Robert M. Schulman</td> <td>31,196</td> <td>Gerald F. Swiss</td> <td>30,113</td> </tr> <tr> <td>Samuel C. Miller, III</td> <td>27,360</td> <td></td> <td></td> <td></td> <td></td> </tr> </table>					William L. Mathis	17,337	Ralph L. Freeland, Jr.	16,110	William C. Rowland	30,888	Peter H. Smolka	15,913	Robert G. Mukai	28,531	T. Gene Dillahunt	25,423	Robert S. Swecker	19,885	George A. Hovanec, Jr.	28,223	Anthony W. Shaw	30,104	Platon N. Mandros	22,124	James A. LaBarre	28,632	Patrick C. Keane	32,858	Benton S. Duffett, Jr.	22,030	E. Joseph Gess	28,510	Bruce J. Boggs, Jr.	32,344	Joseph R. Magnone	24,239	R. Danny Huntington	27,903	William H. Benz	25,952	Norman H. Stepno	22,716	Eric H. Weisblatt	30,505	Peter K. Skiff	31,917	Ronald L. Grudziecki	24,970	James W. Peterson	26,057	Richard J. McGrath	29,195	Frederick G. Michaud, Jr.	26,003	Teresa Stanek Rea	30,427	Matthew L. Schneider	32,814	Alan E. Kopecki	25,813	Robert E. Krebs	25,885	Michael G. Savage	32,596	Regis E. Slutter	26,999	Robert M. Schulman	31,196	Gerald F. Swiss	30,113	Samuel C. Miller, III	27,360				
William L. Mathis	17,337	Ralph L. Freeland, Jr.	16,110	William C. Rowland	30,888																																																																							
Peter H. Smolka	15,913	Robert G. Mukai	28,531	T. Gene Dillahunt	25,423																																																																							
Robert S. Swecker	19,885	George A. Hovanec, Jr.	28,223	Anthony W. Shaw	30,104																																																																							
Platon N. Mandros	22,124	James A. LaBarre	28,632	Patrick C. Keane	32,858																																																																							
Benton S. Duffett, Jr.	22,030	E. Joseph Gess	28,510	Bruce J. Boggs, Jr.	32,344																																																																							
Joseph R. Magnone	24,239	R. Danny Huntington	27,903	William H. Benz	25,952																																																																							
Norman H. Stepno	22,716	Eric H. Weisblatt	30,505	Peter K. Skiff	31,917																																																																							
Ronald L. Grudziecki	24,970	James W. Peterson	26,057	Richard J. McGrath	29,195																																																																							
Frederick G. Michaud, Jr.	26,003	Teresa Stanek Rea	30,427	Matthew L. Schneider	32,814																																																																							
Alan E. Kopecki	25,813	Robert E. Krebs	25,885	Michael G. Savage	32,596																																																																							
Regis E. Slutter	26,999	Robert M. Schulman	31,196	Gerald F. Swiss	30,113																																																																							
Samuel C. Miller, III	27,360																																																																											
<p>and: <u>Leslie A. Mooi, Reg. No. 37,047 and Earl A. Bright II, Reg. No. 37,045</u></p>																																																																												
<p>Address all correspondence to: Gerald F. Swiss, Esq. BURNS, DOANE, SWECKER & MATHIS, L.L.P. P.O. Box 1404 Alexandria, Virginia 22313-1404</p>																																																																												
<p>Address all telephone calls to: <u>Leslie A. Mooi</u> at (415) 854-7400.</p>																																																																												
<p>I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.</p>																																																																												
FULL NAME OF SOLE OR FIRST INVENTOR		SIGNATURE		DATE																																																																								
Daniel Jed Harrison																																																																												
RESIDENCE			CITIZENSHIP																																																																									
8932 151 Street, Edmonton, Alberta T5R 1J2, Canada			Canada																																																																									
POST OFFICE ADDRESS																																																																												
8932 151 Street, Edmonton, Alberta T5R 1J2, Canada																																																																												
FULL NAME OF SECOND JOINT INVENTOR, IF ANY		SIGNATURE		DATE																																																																								
Per Andersson																																																																												
RESIDENCE			CITIZENSHIP																																																																									
Apartment #9 10530 79th Avenue, Edmonton, Alberta T6G 1R8, Canada			Sweden																																																																									
POST OFFICE ADDRESS																																																																												
Apartment #9 10530 79th Avenue, Edmonton, Alberta T6G 1R8, Canada																																																																												
FULL NAME OF THIRD JOINT INVENTOR, IF ANY		SIGNATURE		DATE																																																																								
Paul Li																																																																												
RESIDENCE			CITIZENSHIP																																																																									
			Hong Kong																																																																									
POST OFFICE ADDRESS																																																																												

FULL NAME OF FOURTH JOINT INVENTOR, IF ANY		SIGNATURE		DATE	
Roderick Szarka					
RESIDENCE			CITIZENSHIP		
11712 10th Avenue, Edmonton, Alberta T6J 7A6, Canada			Canada		
POST OFFICE ADDRESS					
11712 10th Avenue, Edmonton, Alberta T6J 7A6, Canada					
FULL NAME OF FIFTH JOINT INVENTOR, IF ANY		SIGNATURE		DATE	
Richard Smith					
RESIDENCE			CITIZENSHIP		
1010 Buchanan Place, Edmonton, Alberta T6R 2A6, Canada			Canada		
POST OFFICE ADDRESS					
1010 Buchanan Place, Edmonton, Alberta T6R 2A6, Canada					
FULL NAME OF SIXTH JOINT INVENTOR, IF ANY		SIGNATURE		DATE	
RESIDENCE			CITIZENSHIP		
POST OFFICE ADDRESS					
FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY		SIGNATURE		DATE	
RESIDENCE			CITIZENSHIP		
POST OFFICE ADDRESS					
FULL NAME OF EIGHTH JOINT INVENTOR, IF ANY		SIGNATURE		DATE	
RESIDENCE			CITIZENSHIP		
POST OFFICE ADDRESS					
FULL NAME OF NINTH JOINT INVENTOR, IF ANY		SIGNATURE		DATE	
RESIDENCE			CITIZENSHIP		
POST OFFICE ADDRESS					